STUDIES ON THE UNFOLDING AND ZINC STATUS OF THERMOLYSIN AND CARBOXYPEPTIDASE A, AND THE DESIGN OF A FRET-QUENCHED PEPTIDE FOR ASSAYING THERMOLYSIN-LIKE PROTEASES

By

Danica Goulet

A thesis submitted in partial fulfillment of the requirements for the degree Master of Science (M.Sc.) in Chemical Sciences

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

© Danica Goulet, 2020
Title of Thesis
Titre de la thèse
Studies On The Unfolding and Zinc Status of Thermolysin and Carboxypeptidase A, and the design of a fret-quenched peptide for assaying thermolysin-like proteases

Name of Candidate
Nom du candidat
Goulet, Danica

Degree
Diplôme
Master of Science

Department/Program
Département/Programme
Chemical Sciences

Date of Defence
Date de la soutenance September 16, 2020

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. Stefan Siemann
(Supervisor/Directeur(trice) de thèse)

Dr. Eric Gauthier
(Committee member/Membre du comité)

Dr. Jeffrey Shepherd
(Committee member/Membre du comité)

Dr. Mazdak Khajehpour
(External Examiner/Examinateur externe)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. Serge Demers
Monsieur Serge Demers

Acting Dean, Faculty of Graduate Studies
Doyen intérimaire, Faculté des études supérieures

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

I, Danica Goulet, hereby grant to Laurentian University and/or its agents the non-exclusive license to archive and make accessible my thesis, dissertation, or project report in whole or in part in all forms of media, now or for the duration of my copyright ownership. I retain all other ownership rights to the copyright of the thesis, dissertation or project report. I also reserve the right to use in future works (such as articles or books) all or part of this thesis, dissertation, or project report. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that this copy is being made available in this form by the authority of the copyright owner solely for the purpose of private study and research and may not be copied or reproduced except as permitted by the copyright laws without written authority from the copyright owner.
Abstract

The chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR) has previously been employed as a probe to determine structural transitions during protein (un)folding in the zinc-dependent anthrax lethal factor protease (LF). Whether PAR can be employed more widely as a zinc accessibility probe (ZAP) to study the unfolding of other zinc proteins remains to be established. Using a combination of intrinsic tryptophan fluorescence spectroscopy and chelator studies, the unfolding pathways and the influence of the protein fold on the metal status were investigated for the zinc proteases thermolysin (TL) and carboxypeptidase A (CPA). These studies revealed considerable differences in the unfolding pathways and the resistance to metal dissociation for these enzymes. In addition, the observations reported herein demonstrate that ZAPs might be of value in future investigations to gain further insight into the mechanisms underlying the metal-mediated misfolding of proteins involved in a variety of neurodegenerative diseases. Finally, this study describes a new fluorescence-quenched heptapeptide (Dabcyl-FKFLGKE-EDANS), which shows the largest specificity constant documented for any TL substrate.
Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor, Dr. Siemann. Your immense knowledge, support, guidance, and patience throughout this project has made my M.Sc. experience a truly excellent one. I also thank my supervisory committee members, Dr. Gauthier and Dr. Shepherd, for their insightful comments and helpful suggestions on this project. In addition, I would like to thank my labmate Tyson Pilkington for preparing samples of concentrated zinc lethal factor, my labmates Usama Fraaz and Carly Zulich for their assistance in the lab, as well as all professors and technicians of the Chemistry and Biochemistry Department at Laurentian University. Last but certainly not least, I thank my parents, brother, and friends for their support and encouragement.
Table of Contents

Abstract ................................................................................................................................. iii
Acknowledgements ................................................................................................................. iv
List of Abbreviations .............................................................................................................. vii
List of Figures ........................................................................................................................ viii
List of Tables .......................................................................................................................... x

1. General Introduction ......................................................................................................... 1
   1.1 Essentiality of zinc ......................................................................................................... 1
   1.2 Zinc in humans ................................................................................................................ 2
   1.3 Properties of zinc ........................................................................................................... 2
   1.4 Zinc-binding sites .......................................................................................................... 3
   1.5 Zinc proteases ................................................................................................................ 7
       1.5.1 Carboxypeptidase A .............................................................................................. 8
       1.5.2 Thermolysin .......................................................................................................... 11
       1.5.3 Anthrax lethal factor ............................................................................................. 14

2. General Hypotheses and Objectives ............................................................................... 21
   2.1 Unfolding and metal binding studies on TL and CPA ................................................. 21
   2.2 Design of a new fluorescent substrate for TL and TL-like proteases ......................... 22

3. Unfolding and Metal Binding Studies on TL and CPA ..................................................... 23
   3.1 Background ................................................................................................................... 23
   3.2 Hypotheses and Objectives .......................................................................................... 26
   3.3 Materials and Methods ................................................................................................. 28
       3.3.1 Chemicals and laboratory equipment ................................................................. 28
       3.3.2 Stock solutions ..................................................................................................... 30
       3.3.3 Enzyme preparation .............................................................................................. 31
       3.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE) ....................................... 31
       3.3.5 Tryptophan fluorescence spectroscopy .............................................................. 34
       3.3.6 ANS fluorescence spectroscopy ............................................................................ 37
       3.3.7 CPA activity assay ............................................................................................... 39
       3.3.8 Reversibility of CPA unfolding ............................................................................ 39
       3.3.9 Zinc release .......................................................................................................... 42
       3.3.10 Zinc accessibility ................................................................................................. 43
       3.3.11 Stopped-flow spectrophotometry ....................................................................... 43

3.4 Results ............................................................................................................................. 45
   3.4.1 Tryptophan fluorescence emission spectra of TL .................................................... 45
   3.4.2 TL autolysis ............................................................................................................. 47
   3.4.3 TL unfolding ............................................................................................................ 50
   3.4.4 Zinc accessibility and release from TL ................................................................. 51
   3.4.5 Tryptophan fluorescence studies on CPA .............................................................. 53
List of Tables

Table 1.1: The affinities of various zinc proteins for Zn$^{2+}$................................................................. 7
Table 3.1: Laboratory equipment.................................................................................................................. 28
Table 3.2: List of chemical reagents and suppliers...................................................................................... 29
Table 3.3: Composition of the polyacrylamide gels. .................................................................................... 32
Table 3.4: Composition of the running buffer (1 L)..................................................................................... 32
Table 3.5: Composition of the Coomassie Blue staining solution (1 L) ..................................................... 33
Table 3.6: Composition of the destaining solution (1 L) ........................................................................... 33
Table 3.7: Summary of thermodynamic parameters and midpoints obtained from the individual and global fits of CPA unfolding................................................................. 57
Table 3.8: The thermodynamic parameters and midpoints derived from the individual and global fits of CPA denaturation in the presence of ANS......................................................... 63
Table 3.9: Summary of C$_{mid}$ values from the unfolding, zinc accessibility, and zinc release studies for LF, TL, and CPA. .................................................................................................... 85
Table 4.1: Analysis of the Dabcyl-FKFLGKE-EDANS hydrolysis products by ESI-MS. ....................... 105
Table 4.2: Hydrolysis of Dabcyl-FKFLGKE-EDANS by various proteases ........................................... 112
Table 4.3: Kinetic parameters of TL-mediated hydrolysis of various peptide substrates. ........... 116
List of Figures

Figure 1.1: Zinc-binding sites in proteins.............................................................. 3
Figure 1.2: Role of water in catalytic zinc-binding sites. ........................................ 4
Figure 1.3: Structure of CPA. ............................................................................... 9
Figure 1.4: Proposed mechanisms of ester hydrolysis by CPA. ............................. 11
Figure 1.5: Structure of TL .................................................................................. 13
Figure 1.6: Proposed catalytic mechanism of TL .................................................. 14
Figure 1.7: Structure of LF. .................................................................................. 15
Figure 1.8: Two proposed catalytic mechanisms of LF. ......................................... 16
Figure 1.9: Atomic model of the PA pore .............................................................. 18
Figure 1.10: Influence of GdnHCl on the fold, metal release and accessibility of LF .............................................................. 19
Figure 1.11: Influence of GdnHCl on the fold, metal release and accessibility of LF .............................................................. 19
Figure 3.1: Trp fluorescence spectra of native and GdnHCl-exposed TL ............... 46
Figure 3.2: Silver stained SDS-PAGE gel of denatured and Amicon-filtered TL .... 48
Figure 3.3: Time dependence of TL unfolding ...................................................... 49
Figure 3.4: SDS-PAGE/silver stain analysis on the time dependence of TL autolysis in the presence of 10 mM Ca^{2+} ............................................................. 50
Figure 3.5: Unfolding profile for TL in the presence of GdnHCl ......................... 51
Figure 3.6: Influence of GdnHCl on the metal release (blue) and accessibility (red) of TL .............................................................................................................. 52
Figure 3.7: Trp fluorescence spectra of CPA.......................................................... 54
Figure 3.8: Time dependence of CPA unfolding .................................................. 54
Figure 3.9: Unfolding profile for CPA in the presence of GdnHCl ....................... 56
Figure 3.10: Global fitting curves of GdnHCl-induced CPA unfolding ................ 59
Figure 3.11: ANS fluorescence spectra of CPA exposed to GdnHCl ................... 61
Figure 3.12: GdnHCl concentration dependence of the ANS fluorescence intensity during the unfolding of CPA ................................................................. 62
Figure 3.13: Fractional occupancies of the native, intermediate, and unfolded states of CPA. ................................................................. 64
Figure 3.14: Trp fluorescence spectra of unfolded and partially renatured CPA ................................................................. 66
Figure 3.15: Refolding of CPA from the unfolded and intermediate states ............................. 67
Figure 3.16: ANS fluorescence spectra of unfolded and partially renatured CPA ................................................................. 69
Figure 3.17: Trp fluorescence spectra of CPA assessing reversibility (I → N) and quenching by ANS ............................................................................................... 71
Figure 3.18: Influence of GdnHCl on the metal release (blue) and accessibility (red) of CPA ................................................................. 72
Figure 3.19: Stopped-flow UV-Vis spectrophotometric determination of the progress of Zn^{2+} chelation by PAR ..................................................................................... 75
Figure 4.1: Structures of common FRET systems .................................................. 90
Figure 4.2: Overall structure of the Dabcyl-FKFLGKE-EDANS peptide ............. 92
Figure 4.3: Schematic representation of the fluorescence enhancement by Dabcyl-FKFLGKE-EDANS hydrolysis ................................................................. 102
Figure 4.4: Reaction progress curve for TL-mediated hydrolysis of Dabcyl-FKFLGKE-EDANS. 103
Figure 4.5: Mass spectrum of Dabcyl-FKFLGKE-EDANS ................................ 104
Figure 4.6: Mass spectrum of Dabcyl-FKFLGKE-EDANS after TL-catalyzed hydrolysis .......... 106
Figure 4.7: Dependence of fluorescence intensity on the concentration of hydrolyzed Dabcyl-FKFLGKE-EDANS................................................................. 107
Figure 4.8: Michaelis-Menten plots of TL-mediated Dabcyl-FKFLGKE-EDANS hydrolysis at high (10 mM) and low (50 µM) CaCl₂ concentrations................................................. 108
Figure 4.9: pH dependence of Dabcyl-FKFLGKE-EDANS hydrolysis by TL.................................................. 109
Figure 4.10: Temperature dependence of TL-catalyzed Dabcyl-FKFLGKE-EDANS............................................. 111
Figure 4.11: Michaelis-Menten plot of the dispase-mediated cleavage of Dabcyl-FKFLGKE-EDANS.......................................................... 113
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>Ångström</td>
</tr>
<tr>
<td>Abz</td>
<td>2-Aminobenzoyl</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMT buffer</td>
<td>Three-component buffer consisting of acetic acid, MES, and TRIS</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilinonaphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>Ar</td>
<td>4-Methoxyphenyl</td>
</tr>
<tr>
<td>Bim</td>
<td>1,7-Dioxo-2,5,6-trimethyl-1H,7H-pyrazolo[1,2-α]-pyrazol-3-yl-methyl</td>
</tr>
<tr>
<td>BHQ2</td>
<td>Black hole quencher 2</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>CF</td>
<td>Carboxyfluorescein</td>
</tr>
<tr>
<td>CFSE</td>
<td>Crystal field stabilization energy</td>
</tr>
<tr>
<td>C_{mid}</td>
<td>Midpoint concentration</td>
</tr>
<tr>
<td>CPA</td>
<td>Carboxypeptidase A</td>
</tr>
<tr>
<td>CPY</td>
<td>Carboxypeptidase Y</td>
</tr>
<tr>
<td>cps</td>
<td>Counts per second</td>
</tr>
<tr>
<td>Dabcyl</td>
<td>4-(4′-N,N-Dimethylaminophenyl)azobenzoic acid</td>
</tr>
<tr>
<td>Dabsyl</td>
<td>Dimethylaminobenzenesulfonyl</td>
</tr>
<tr>
<td>DBDY</td>
<td>N,N′-DiBoc-dityrosine</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Dnp</td>
<td>2,4-Dinitrophenyl</td>
</tr>
<tr>
<td>Dns</td>
<td>5-(Dimethylamino)-naphthalenyl-1-sulfanyl</td>
</tr>
<tr>
<td>Dpa</td>
<td>N^3-(2,4-Dinitrophenyl)-L-2,3-diaminopropionyl</td>
</tr>
<tr>
<td>EDANS</td>
<td>N-(2-Aminoethyl)aminonaphthalene-5-sulfonic acid</td>
</tr>
<tr>
<td>EF</td>
<td>Edema factor</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FAGLA</td>
<td>N-Furlyacyloylglycyl-L-leucinamide</td>
</tr>
<tr>
<td>FI</td>
<td>Fluorescence intensity</td>
</tr>
<tr>
<td>f_i</td>
<td>Intermediate fraction</td>
</tr>
<tr>
<td>f_N</td>
<td>Native fraction</td>
</tr>
<tr>
<td>f_U</td>
<td>Unfolded fraction</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>Fua</td>
<td>N-Furlyacyloyl</td>
</tr>
<tr>
<td>GdnHCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>I</td>
<td>Intermediate state</td>
</tr>
<tr>
<td>IFE</td>
<td>Inner filter effect</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>k_{cat}</td>
<td>Turnover number</td>
</tr>
<tr>
<td>k_{cat} / K_M</td>
<td>Catalytic efficiency</td>
</tr>
</tbody>
</table>
$K_d$ Dissociation constant
kDa Kilodalton
$K_M$ Michaelis constant
LF Lethal factor
$m$ Denaturant dependent of $\Delta G^0$
MAPKK Mitogen-activated protein kinase kinase
Mca (7-Methoxycoumarin-4-yl)acetyl
MES 2-(N-Morpholino)ethanesulfonic acid
MG Molten globule
MT Metallothionein
m/z Mass-to-charge ratio
N Native state
Nba 4-Nitrobenzylamide
PA Protective antigen
PAR 4-(2-Pyridylazo)resorcinol
PDB ID Protein databank identification code
pH Potential of hydrogen
$pK_a$ Acid dissociation constant
R Gas constant
S Substrate
SASA Solvent-accessible surface area
s.d. Standard deviation
SDS Sodium dodecyl sulfate
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD1 Cu/Zn superoxide dismutase
T Temperature
TEMED $N-N-N'-N'$-Tetramethylethylenediamine
TL Thermolysin
Tris Tris(hydroxymethyl)aminomethane
TS Transition state
U Unfolded state
UV Ultra-violet
v/v Volume by volume
$v_o$ Initial velocity
$v_{max}$ Maximum velocity
w/v Weight by volume
ZAP Zinc accessibility probe
ZAPM $N$-Carbobenzoxy-L-aspartyl-L-phenylalaninemethyl ester
4MeO2NA 4-Methoxy-2-naphthylamine
$\Delta G^0$ Standard Gibbs free energy of unfolding in the absence of denaturant
$\lambda_{em}$ Emission wavelength
$\lambda_{exc}$ Excitation wavelength
$\lambda_{max}$ Emission/absorption maximum wavelength
1. General Introduction

1.1 Essentiality of zinc

In 1869, Raulin described, for the first time, the essentiality of zinc for the growth of the mold Aspergillus niger [1]. Zinc was later proven to be essential for the growth of rats in 1934 [1]. Around thirty years later, in 1962, the same conclusion was reached for plants and humans [2]. The transition metal has been shown to be required for the growth, development, and differentiation of microorganisms, plants, and animals [3]. In animals, zinc is involved in many aspects of the immune system, being essential for the development and function of cells mediating innate immunity (i.e., neutrophils, macrophages, and natural killer cells) [4].

For an adult human, dietary intake of approximately 15 mg of zinc per day is required to maintain good health and immune system function [5]. As is generally the case with all essential metals, insufficient uptake of zinc results in symptoms of deficiency while an excess leads to toxicity. Zinc deficiency in humans has affected nearly two billion people in developing countries [4]. Deficiency of this essential metal has severe effects in humans, including growth retardation, male hypogonadism, immune dysfunction, decreased wound healing, infertility, and cognitive impairment [2, 4]. On the other hand, lethargy, nausea, diarrhea, altered immune system function, and symptoms of copper deficiency are often observed as a result of excess zinc in humans [6]. Copper deficiency and zinc toxicity go hand in hand due to the upregulation of metallothionein (MT), a cysteine-rich protein involved in metal detoxification in response to elevated cellular zinc levels [7]. MT has a high affinity for copper, and therefore will not only reduce toxic levels of zinc, but will also deplete cellular copper pools [8].
1.2 Zinc in humans

Between 2 and 4 g of zinc is distributed throughout the human body, making it the second most abundant transition metal, after iron [9]. The metal is present in the brain, kidney, skin, and liver, with muscle and bone accounting for nearly 90% of the body zinc content [8, 10]. In 1940, Keilin and Mann discovered the first zinc metalloenzyme, carbonic anhydrase (CA) [11]. Since then, over 300 enzymes (spanning all 7 enzyme classes [12]) requiring zinc for catalytic activity have been discovered [6]. It has been estimated that zinc plays a role in about 10% of proteins in the human proteome, and is required for function and structural integrity in ~2000 transcription factors [2, 6, 8]. Zinc ions (Zn\(^{2+}\)) can also act as both a neurotransmitter (first messenger in cell-to-cell communication), and as an intracellular signalling molecule, where it functions as the secondary messenger required for DNA synthesis [13].

1.3 Properties of zinc

Under biological conditions, Zn\(^{2+}\) is redox inert, and behaves as a strong, borderline Lewis acid [14, 15]. Due to its lack of crystal field stabilization energy (CFSE), a consequence of its filled \(d\) orbital shell (\(d^{10}\)), it has a flexible coordination geometry, with a coordination number of four (tetrahedral geometry) being most common in metalloenzymes [15]. Flexibility in coordination geometry usually leads to rapid substrate binding and product release [16]. Additionally, as a borderline Lewis acid, the 3\(d\) transition metal can bind a vast range of biological ligands (i.e., hard, soft, and borderline Lewis bases), and form very stable metal-protein complexes. In fact, of the 3\(d\) metal ions, only Cu\(^{2+}\) is shown to bind ligands more strongly, a feature reflected in the Irving-Williams series (Mn\(^{2+}\) < Fe\(^{2+}\) < Co\(^{2+}\) < Ni\(^{2+}\) < Cu\(^{2+}\) > Zn\(^{2+}\)) [14, 17].
1.4 Zinc-binding sites

The zinc ion is an essential cofactor for the biological function of many metalloenzymes. The metal-binding sites in zinc proteins can be categorized into four distinct zinc binding site classes, which include catalytic, structural, co-catalytic, and protein interface (see Figure 1.1) [18]. More recently, this classification has been expanded to also include clustered and transport-related zinc sites [19]. Histidine, glutamate, aspartate, and cysteine residues are the most frequently observed amino acid ligands in these sites [20].

![Figure 1.1: Zinc-binding sites in proteins.](image)

(A) Catalytic, (B) structural, (C) co-catalytic, and (D) protein interface sites. The protein interface sites can be further divided into sites in which Zn$^{2+}$ stabilizes homodimers (left) and in which the metal binds to two subunits to form a catalytic site (right). This figure was modified from [18] with permission from Springer Nature (license number: 4912020453169).

The catalytic zinc binding site is found in most zinc enzymes, and contains one zinc ion coordinated to three ligands, and one water molecule acting as a nucleophile in catalysis (Figure 1.1a) [18]. As shown in Figure 1.2, the water molecule can be activated for catalysis either by direct ionization (observed in CA), polarization by a neighbouring amino acid residue acting as a general acid/base (documented for carboxypeptidase A, CPA), by displacement (observed with alkaline phosphatase), or by expansion of the coordination sphere upon interaction with a
substrate [21, 22]. A coordination number of usually 4 or 5 in a distorted tetrahedral or trigonal bipyramidal geometry is typical for this binding site [18]. Enzymes with a catalytic zinc binding site include many hydrolases including the aforementioned enzymes, thermolysin (TL), alcohol dehydrogenase, and anthrax lethal factor (LF) [23].

![Figure 1.2: Role of water in catalytic zinc-binding sites.](image)

In contrast to catalytic zinc sites, the structural zinc site stabilizes the tertiary structure of a protein (see Figure 1.1b) [15]. In this site, the metal is usually tetrahedrally coordinated to four ligands [15]. The structural zinc-binding site lacks a metal-bound water molecule, and cysteine is the most predominant amino acid ligand, with histidine also being commonly observed in these sites [18]. Zinc fingers (DNA/RNA-binding zinc proteins) including GATA4, KLF4, and ZFP36, are amongst the many proteins that require the metal for structural stability [24, 25].
Co-catalytic zinc binding sites are found in enzymes containing two or more zinc (and/or other metal ions) in the active site (see Figure 1.1c) [15, 18]. The metal ions in this site are bound in close proximity to one another. Aspartate, glutamate, histidine, a water molecule, and a carboxylated lysine have previously been shown to bridge metal ions in co-catalytic sites, with aspartate being the most frequently encountered bridging ligand [18]. In co-catalytic zinc sites, one metal ion has a role in catalytic function, and the other metal(s) is responsible for enhancing catalysis. For example, in Cu/Zn superoxide dismutase (SOD1), the copper ion undergoes oxidation/reduction during catalysis, whereas the zinc ion is non-catalytic, and is thought to maintain the structure of the active site [23]. Other examples of enzymes with a co-catalytic zinc site include alkaline phosphatase, purple acid phosphatase, phospholipase C, nuclease P1, and leucine aminopeptidase [15].

Another class of zinc-binding sites is termed protein interface or interfacial (see Figure 1.1d). Here, the metal coordinates to at least two polypeptides, where histidine, glutamate, aspartate, and less commonly cysteine, supply the ligands to these sites [20]. The zinc ion can influence the quaternary structure of a protein, by either inducing dimer- or trimerization of the same protein, or by linking two different proteins through the intermolecular ligands [18]. An example of the former case is endothelial nitric oxide (eNOS) synthase, where the zinc ion is tetrahedrally coordinated to a pair of cysteine residues of two identical monomers, ultimately leading to the formation of a dimer [18]. In addition, the T-cell co-receptors CD4 and CD8, and the protein tyrosine phosphatase Lck, were identified and characterized to bind via Zn$^{2+}$ coordination [26]. A catalytic zinc ion can also be observed in this class of zinc sites. For example, the zinc site of γ-carbonic anhydrase from Methanosarcina thermophila lies at the interface
between the three subunits of the trimeric enzyme, where each zinc ion coordinates to three histidine residues and two water molecules in a trigonal bipyramidal coordination geometry [18].

More recently, the clustered and transport-related sites have been included as classes of zinc-binding sites [19]. MTs are the prime example of the clustered zinc-binding site. They are involved in metal storage and detoxification, and harbour two sites containing four and three zinc ions (for a total of up to 7) [19, 27]. In GAL4, a protein harbouring a DNA-binding domain, two zinc ions are bound to six cysteine residues, thus forming a dizinc/thiolate cluster [28]. The final class of zinc sites is associated to zinc transport [19]. Zinc transporters, such as Zrt/Irt-like protein (ZIP) and the ZnT proteins, both bind Zn\(^{2+}\) to facilitate its transport across membranes [19]. YiiP, a ZnT protein homolog in *E. coli*, harbours four zinc-binding sites, where the metal ion is tetrahedrally coordinated to three aspartate residues and one histidine residue [19]. Additionally, human serum albumin (HSA) is thought to transport zinc in plasma [29], and the site having the highest affinity for Zn\(^{2+}\) has a tetrahedral geometry, and is comprised of two histidine residues, an aspartate residue, and a water molecule [30].

The concentration of free cellular zinc is generally low (in the picomolar range). However, the concentration varies in certain organisms and cell types [31]. The majority of cellular zinc is bound to cytosolic zinc-dependent proteins possessing a high affinity for the divalent metal. A zinc affinity in the pico-to-femtomolar range is required for a zinc-dependent protein, otherwise, the protein would lose the metal ion as well as its functionality in the cytosol [31]. The dissociation constants \((K_d)\) for zinc have been experimentally determined for many zinc-dependent proteins and enzymes, with values ranging from the femtomolar to the nanomolar.
range. As Table 1.1 shows, the magnitude of the $K_d$ values critically depends on the type of the zinc-binding sites, and is fine-tuned by the nature of the metal ion’s ligands.

**Table 1.1: The affinities of various zinc proteins for Zn$^{2+}$.**

<table>
<thead>
<tr>
<th>Zinc protein</th>
<th>Zinc-binding site</th>
<th>$K_d$ (M)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthrax lethal factor</td>
<td>Catalytic</td>
<td>$1.2 \times 10^{-12}$</td>
<td>[32]</td>
</tr>
<tr>
<td>Carbonic anhydrase II, human</td>
<td>Catalytic</td>
<td>$1 \times 10^{-12}$</td>
<td>[33]</td>
</tr>
<tr>
<td>Carboxypeptidase A, bovine</td>
<td>Catalytic</td>
<td>$3.2 \times 10^{-11}$</td>
<td>[34]</td>
</tr>
<tr>
<td>Dispase (<em>B. subtilis</em>)</td>
<td>Catalytic</td>
<td>$2.2 \times 10^{-13}$</td>
<td>[35]</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>Transport</td>
<td>$3.2 \times 10^{-8}$</td>
<td>[36]</td>
</tr>
<tr>
<td>Metallothionein (MT3)</td>
<td>Clustered</td>
<td>$3.2 \times 10^{-12}$ (7 sites)</td>
<td>[37]</td>
</tr>
<tr>
<td>Neural zinc finger factor 1</td>
<td>Structural</td>
<td>$1.26 \times 10^{-10}$</td>
<td>[38]</td>
</tr>
<tr>
<td>Cu/Zn superoxide dismutase</td>
<td>Co-catalytic</td>
<td>$4.0 \times 10^{-14}$</td>
<td>[39]</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>Catalytic</td>
<td>$2.4 \times 10^{-13}$</td>
<td>[35]</td>
</tr>
</tbody>
</table>

**1.5 Zinc proteases**

Metalloproteases are involved in many biological processes such as embryonic development, the processing of peptide hormones, the release of cytokines and growth factors, cell adhesion and migration, metabolism of antibiotics, viral polyprotein processing, and many more [40]. Therefore, numerous diseases can arise due to the aberrant activities of these essential metalloproteases, including arthritis, cancer, cardiovascular diseases, nephritis, disorders of the central nervous system, fibrosis, and infection [40]. The majority of metalloproteases require the divalent Zn$^{2+}$ ion as a cofactor [41]. In some cases, these enzymes can contain a cobalt, manganese, or nickel ion instead of Zn$^{2+}$ [40]. As previously mentioned in section 1.4, zinc ions in catalytic sites are usually tetrahedrally coordinated to three amino acid residues (typically in a combination of histidine, glutamate, aspartate, and cysteine), and a water molecule acting as the nucleophile in the hydrolysis of the peptide bond [18]. Histidine is the
most frequently observed residue in these sites, followed by glutamate, and then aspartate and cysteine [15, 40]. Examples of zinc proteases with a zinc binding motif containing three histidine residues and a water molecule include adenosine deaminase, astacin, and metallo-β-lactamases [15]. One of the earliest identified and best studied zinc proteases, carboxypeptidase A (CPA), contains a zinc ion coordinated to two histidine residues, a glutamate residue, and a water molecule in the active site [23], with the metal-bound glutamate in the active site partially neutralizing the charge of the divalent ion.

A large number of metallopeptidases harbour the metal ion binding motif HExxH, where the two histidine residues serve as ligands, x is any amino acid, and the glutamate of the motif serves as a general base in the catalytic mechanism [40, 42]. The thermolysin-like zinc binding consensus motif, $H\text{ExxH}(X_n)E$, where n is at least 14 residues and the underlined residues are coordinating the catalytic zinc ion, is characteristic of members of the gluzincin clan [41, 43]. The metalloendopeptidases thermolysin and anthrax lethal factor, as well as tetanus and botulinum neurotoxins, are members of this family.

1.5.1 Carboxypeptidase A

Bovine carboxypeptidase A (CPA) is one of the earliest identified zinc metalloenzymes, and is the most studied protein amongst all zinc proteases [44]. In fact, this enzyme serves as a prototype for a large family of metalloproteases, the M14 metallopeptidase family [45]. Biologically, this pancreatic exopeptidase catalyzes the degradation of food proteins, where it preferentially removes C-terminal amino acid residues with aromatic or branched sidechains [44]. CPA has also been used for commercial applications, where it is utilized for the hydrolysis
of cheese whey protein [46], and the production of protein hydrolysates free of aromatic amino acid residues [47]. Enzymatic protein hydrolysates have been shown to improve and/or modify the functional properties of food products, and phenylalanine-free protein hydrolysates can be ingested by patients with phenylketonuria [47].

CPA was one of the first proteins whose structure was determined by X-ray diffraction with high-resolution [48]. Therefore, the enzyme has been used as a model protein to study the structure and catalytic mechanism of zinc enzymes [45]. The X-ray studies revealed that this 34.5 kDa metalloexopeptidase consists of a single polypeptide chain of 307 amino acid residues with a single zinc ion in the active site [48, 49]. Unlike thermolysin and anthrax lethal factor (described below), CPA does not contain an HExxH motif. The divalent zinc cofactor is coordinated to Glu72, the $N_e2$ nitrogen atoms of His69 and His196, and a water molecule serving as the nucleophile in the peptide bond cleavage reaction (see Figure 1.3) [44]. The water molecule in the active site is hydrogen bonded to Glu270, a residue required for enzymatic activity [49].

---

**Figure 1.3: Structure of CPA.** (A) The overall fold of CPA (PDB ID: 5CPA) [50]. The catalytic $Zn^{2+}$ ion is depicted in orange. (B) The catalytic $Zn^{2+}$ ion (orange) is coordinated to His69, Glu72, His196, and a water molecule in a distorted trigonal bipyramidal geometry. Both Glu270 and Arg127 contribute to the catalytic mechanism of CPA.
Despite extensive research, the catalytic mechanism of CPA remains controversial. Two possible reaction pathways (investigated by ester hydrolysis) by CPA have been proposed, the promoted-water pathway and the nucleophilic pathway (see Figure 1.4) [51]. In the promoted-water pathway, Glu270 serves as both a general base (in the nucleophilic addition step) and as a general acid (in the second elimination step) (see Figure 1.4A) [51]. Initially, Glu270 acts as a general base by activating the zinc-bound water molecule, and by accepting a water-derived proton. The residue then acts as a general acid by transferring the proton to the leaving oxygen (for ester hydrolysis) or the nitrogen group (for amide hydrolysis) [51]. Alternatively, in the nucleophilic pathway, the carboxylate side chain of Glu270 attacks the carbonyl carbon atom of the scissile bond, which is then hydrolyzed by an incoming water molecule in the following steps (see Figure 1.4B) [51]. Apart from Glu270, several residues including Arg127, Tyr198, Asn144, Arg145, Tyr248, and Phe279 have been shown to indirectly contribute to the catalytic function of CPA, as well as possibly aid in the binding of the substrate to the enzyme [51-53]. Additionally, the loss of activity is directly proportional to the loss of catalytic zinc ion. However, the activity is regained by the addition of the metal to the apoenzyme [54]. Moreover, CPA is inhibited by excess zinc, in which the metal binds to a second site (i.e., the inhibitory site) in the protein, a feature commonly seen in zinc enzymes [55, 56].
Figure 1.4: Proposed mechanisms of ester hydrolysis by CPA. (A) The promoted-water pathway involves the attack of the carbonyl carbon atom by the zinc-bound water molecule (activated by Glu270). (B) Alternatively, the carboxylate side chain of Glu270 attacks the scissile carbonyl carbon in the nucleophilic pathway. ES = enzyme-substrate complex; TS = transition state; TI = tetrahedral intermediate; AE = acyl-enzyme intermediate; EP = enzyme-product complex. This figure was taken from [51] with permission from the American Chemical Society via RightsLink (Copyright Clearance Center).

1.5.2 Thermolysin

Thermolysin (TL), a thermostable zinc-dependent endopeptidase from Bacillus thermoproteolyticus [57], is the prototype of the M4 family of metallopeptidases [58, 59]. Many members of this family possess considerable potential for industrial and therapeutic applications [60]. Indeed, TL is utilized industrially in the production of the synthetic sweetener aspartame [61, 62], and as a digestive enzyme to generate peptide fragments for sequencing [60]. TL variants have also been developed as a protease component in liquid detergents [63]. Other proteases of
the M4 family including λ-toxin, coccolysin, pseudolysin, and vibriolysin have been implicated as virulence factors in a number of diseases, and they therefore represent viable therapeutic targets [60].

The structure of TL, the best studied member of the M4 family, was solved in 1972 by Matthews and colleagues [64, 65]. The X-ray crystallographic analysis revealed the 34.3 kDa protein to bind up to four calcium ions and a single zinc ion in the active site (see Figure 1.5) [66-68]. While the zinc ion plays a critical role in the catalytic mechanism of TL [69], the calcium ions are believed to serve a structural role by stabilizing the fold of the enzyme [70-72]. The zinc ion in the active site is coordinated to two histidine residues (His142 and His146), a glutamate residue (Glu166), and a water molecule (see Figure 1.5B) [66]. The Glu166 residue was found to bind the metal in a bidentate fashion, with the zinc-bound water molecule occupying two alternative positions (2 Å apart), either binding to Glu143 or to His231 (see Figure 1.5B) [68]. The residues coordinating the zinc ion, and Glu143, form the thermolysin-like zinc binding consensus motif, HExxH(Xn)E.

TL’s substrate specificity has been extensively studied, revealing the amino acid residues N-terminal (P1; according to the definition by Schechter and Berger [73]) and C-terminal (P1’) to the cleavage site to be the most important determinants of enzyme specificity [57]. TL has a strong preference for hydrophobic/bulky amino acid residues (e.g., Phe, Ile, Leu, Val) at the P1’ position, and a Phe residue at the P1 position [74].
Figure 1.5: Structure of TL. (A) The overall structure of TL (PDB ID: 1LNF) [68]. TL contains one catalytic zinc ion (orange) and four calcium ions (magenta) for structural stability. The Ca1,2 double site is located near the zinc ion. (B) TL active site. The divalent zinc ion is coordinated to His142, His146, Glu166 (binding bidentately), and a water molecule serving as the nucleophile. The water molecule bound to Zn$^{2+}$ can occupy two mutually exclusive positions, either hydrogen bonded to Glu143 or to His231. The image in (B) was taken from [75] with permission from the American Chemical Society via RightsLink (Copyright Clearance Center).

The proposed mechanism of TL is shown in Figure 1.6 [76]. The two roles of the catalytic zinc ion include polarization of the carbonyl group, as well as facilitating deprotonation of the water molecule [76]. The Glu143 residue within the HExxH motif serves as a general base in the catalytic mechanism by activating the zinc-bound water nucleophile. The hydrolysis begins with the activated water molecule nucleophilically attacking the carbonyl carbon atom. The side chains of both Tyr157 and His237 provide stabilization for the tetrahedral transition state, and Glu143 shuttles the proton from the water molecule to the nitrogen leaving group [76].
Figure 1.6: Proposed catalytic mechanism of TL. Both the catalytic zinc and Glu143 activate the zinc-bound water ligand. The water molecule serves as a nucleophile and attacks the carbonyl carbon atom. The Glu143 residue acts as a general base, by accepting the proton from the activated water molecule before shuttling it to the nitrogen leaving group. The side chains of Tyr157 and His231 stabilize the transition state (TS). This figure was modified from [76] with permission from the American Chemical Society via RightsLink (Copyright Clearance Center).

1.5.3 Anthrax lethal factor

Anthrax lethal factor (LF) is a zinc-dependent endopeptidase and is one of the three protein components of the anthrax toxin secreted by Bacillus anthracis, along with edema factor (EF, 89kDa) and protective antigen (PA, 83 kDa) [77, 78]. PA is a pore-forming protein, and mediates the translocation of LF and EF from the endosome to the cytosol [79]. LF primarily targets signalling proteins, thus impairing various downstream signalling pathways. While EF is a calmodulin-activated adenyl cyclase involved in the dysregulation of water homeostasis [80], LF is known to cleave and remove the N-terminal segments of almost all types of mitogen-activated protein kinase kinases (MAPKKs) in humans (isoforms 1 – 4, 6 and 7), and of NOD-like receptor protein 1 (Nlrp1) [81, 82].

LF contains four domains, with the catalytic zinc site located in domain IV (see Figure 1.7) [42]. As previously mentioned, LF contains the prototypical thermolysin-like HExxH(Xₙ)E active site binding motif. The catalytic zinc ion is coordinated tetrahedrally to two histidine residues
(His686 and His690), a glutamate residue (Glu735, downstream of the HExxH motif), and a water molecule (see Figure 1.7) [42].

![Figure 1.7: Structure of LF.](image)

**Figure 1.7: Structure of LF.** (A) The overall structure of LF (PDB ID: IJ7N) [42]. LF contains four domains. Domain I is responsible for the binding of PA. Domains II, III, and IV form the substrate binding pocket. The catalytic zinc in domain IV is depicted in yellow. (B) LF active site. The divalent zinc ion (green) is coordinated tetrahedrally to His686, His690, Glu735, and a water molecule. Glu687 and Tyr728 are hydrogen bonded to the water molecule, and are required for catalytic activity.

The two proposed mechanisms of MAPKK hydrolysis are shown in Figure 1.8 [83, 84]. In the first proposed mechanism, the Glu687 residue of the HExxH motif acts as a general base and activates the zinc-bound water molecule required for hydrolytic cleavage (see Figure 1.8A) [42]. The zinc-bound water molecule nucleophilically attacks the carbonyl carbon atom of the scissile peptide bond in the MAPKK substrate, and Tyr728 is thought to be involved in the
Figure 1.8: Two proposed catalytic mechanisms of LF. (A) Residues highlighted in blue are part of the HExxH motif, whereas the residues shown in red are located downstream of the conserved motif. In the mechanism, Glu687 acts as a general base and activates the water molecule, whereas Tyr728 acts as a general acid and stabilizes the amino group of the leaving amino acid. On the other hand, an alternate mechanism suggests that the $\text{Zn}^{2+}$ is coordinated to two water molecules (hydrogen bonded to Glu687 and Tyr728). The alternate mechanism proposes that Tyr728 is responsible for the formation of the enzyme-substrate complex and the stabilization of the carbonyl oxygen atom, whereas Glu687 assists in the deprotonation and orientation of the second water molecule, and delivers a proton to the amine leaving group. The image in (A) was taken from [83] with permission from Elsevier (license number: 4912031212789). The image in (B) was kindly provided by Dr. Stefan Siemann.
protonation/stabilization of the amine leaving group [42, 84]. A recent report proposes an alternate mechanism, where the catalytic zinc ion is coordinated to two water molecules, which are hydrogen bonded to Glu687 and to Tyr728 (see Figure 1.8B) [84]. In this proposed mechanism, the water molecule interacting with Tyr728 is displaced upon the addition of substrate (thus leading to the formation of the enzyme-substrate complex), and Tyr728 stabilizes the increased negative charge on the carbonyl oxygen atom [84]. In addition, Glu687 assists in the deprotonation and orientation of the remaining water molecule towards the carbonyl carbon of the scissile peptide bond, and later delivers a proton to the amine leaving group [84].

As previously mentioned, PA is responsible for LF/EF translocation from the endosome to the cytosol [79]. The PA pore channel is extremely narrow (see Figure 1.9). Therefore, LF is required to unfold to a single polypeptide chain during its transit from the endosome to the cytosol. It is likely that the metal-binding motif is disrupted during this process, since the metal is anchored to residues from two separate helices in domain IV (see Figure 1.7). However, whether Zn$^{2+}$ completely dissociates from LF during translocation, or whether it is somehow cotranslocated, is unknown. If LF were to arrive in the cytosol as an apoprotein, the enzyme would have to be reconstituted with zinc in the cytosol. This is a non-trivial process, however, since free cytosolic zinc levels are extremely low [31].
Figure 1.9: Atomic model of the PA pore. (A) Top and (B) side views of the PA pore channel. PA is a heptamer and forms a 14-stranded transmembrane β-barrel. The structure has 4 domains and is 180 Å long and 160 Å wide. Domain I contains a LF/EF binding site at the entrance of the PA pore. A constriction site of only 6 Å in diameter is present in the channel. This figure was modified from [85] with permission from Springer Nature (license number: 4912031392761).

Although the fate of LF’s Zn$^{2+}$ ion remains to be determined, the enzyme’s fold has previously been investigated using a combination of chelator and tryptophan fluorescence studies at pH 7.4 [86]. These studies revealed LF to unfold in the presence of relatively low concentrations of the denaturant guanidine hydrochloride (GdnHCl), with a midpoint for the 2-state (native → unfolded; N → U) unfolding transition occurring at 0.5 M (see Figure 1.10, blue trace) [86]. It was found that LF was capable of retaining its catalytic zinc ion even after undergoing the conformational change from the native to the unfolded state. Indeed, a concentration of 3.0 M GdnHCl was required to release half of the enzyme’s zinc ions (see Figure 1.10, green trace) [86].
Figure 1.10: Influence of GdnHCl on the fold, metal release and accessibility of LF. Tryptophan fluorescence spectroscopy was used to monitor LF unfolding (blue). The release of zinc from LF (green) and the accessibility of the zinc ion to chelation by PAR (red) were also assessed. The concentration midpoints are shown as dotted, vertical lines. This figure was kindly provided by Dr. Stefan Siemann.

In addition to metal release, metal accessibility studies were performed by exposing the enzyme directly to 4-(2-pyridylazo)resorcinol (PAR, a chromophoric chelator used extensively in investigations on zinc and other metalloproteins to elucidate their metal requirement (see Figure 1.11A) [87-89]) following incubation with the denaturant [86]. Exposing LF directly to PAR allows the chelator to enter the enzyme’s active site to potentially form an enzyme:Zn:PAR ternary complex (see Figure 1.11B) [90]. As shown in Figure 1.10 (red trace), chelation studies with PAR revealed an additional transition (with a midpoint concentration of 1.0 M) distinct from those observed by Trp fluorescence and Zn\(^{2+}\) release measurements. This new transition likely arises from structural changes in LF (i.e., the conversion of a PAR-inaccessible (closed) state to a PAR-
accessible (open) form as shown in Figure 1.11B), thus allowing PAR to gain access to the active site prior to binding and removing the zinc ion. Therefore, PAR is thought to gain access to the LF’s active site once in a denaturant-induced accessible (open) state. Whether such a transition occurs in other zinc proteins, and whether PAR can be employed as a zinc accessibility probe (ZAP) to study protein folding, remains to be established.

Figure 1.11: Structure of PAR and a model of zinc accessibility. (A) The structure of PAR and the Zn$^{2+}$-PAR complex. M$^{2+} = Zn^{2+}$. (B) In the absence or at low concentrations of denaturant, the enzyme is in a PAR-inaccessible (closed) state. Increasing concentrations of the denaturant leads to a transition to an accessible (open) state, in which the position of the equilibrium is determined by the concentration of the denaturant. In the open state, PAR can gain access to the enzyme’s active site and chelate the zinc ion, thus forming an enzyme:Zn:PAR ternary complex. Once the enzyme is demetallated, the free Zn$^{2+}$-PAR complex is capable of binding another PAR molecule to form a complex of 2:1 ligand-metal stoichiometry, if the chelator is in excess.
2. General Hypotheses and Objectives

The work described in this thesis is divided into two parts. The first part (outlined in section 3) is dedicated to studies on the unfolding and metal binding capacity of TL and CPA. The second part describes investigations on a new FRET-quenched peptide to assess the activity of TL and TL-like proteases, and is presented in section 4.

2.1 Unfolding and metal binding studies on TL and CPA

As mentioned previously, studies on the influence of GdnHCl on the structure of LF revealed the enzyme to unfold via a two-state (native → unfolded) mechanism, and to be capable of retaining its zinc ion in the unfolded state [86]. Furthermore, the protein’s metal ion was found to become accessible to chelation by the chromophoric chelator PAR at GdnHCl concentrations in between those required to unfold LF (as assessed by intrinsic Trp fluorescence spectroscopy) and to induce the spontaneous release of Zn$^{2+}$ [86, 90]. Whether similar “accessibility” transitions occur in other zinc proteins is currently unknown.

In section 3, the possibility of using PAR as a zinc accessibility probe (ZAP) to study denaturant-induced structural transitions in TL and CPA (both considered to be amongst the most studied zinc proteases) will be explored. In a fashion similar to that described for LF, the GdnHCl-induced unfolding transitions (for both enzymes) will be investigated by Trp fluorescence spectroscopy, whereas the denaturant-dependence of Zn$^{2+}$ accessibility and spontaneous release will be assessed using PAR. This study is therefore aimed at determining the general applicability of PAR as a ZAP to investigate zinc protein (un/mis)folding.
2.2 Design of a new fluorescent substrate for TL and TL-like proteases

The most common substrate to assess the activity of TL and its relatives is \( N \)-furylacryloylglycyl-L-leucinamide (FAGLA). Limitations associated with this peptide include a low solubility in buffered media [91], as well as a very small change in the extinction coefficients at 345 and 322 nm (\( \Delta \varepsilon_{345\text{ nm}} = 317 \text{ M}^{-1} \text{ cm}^{-1} \), \( \Delta \varepsilon_{322\text{ nm}} = 2300 \text{ M}^{-1} \text{ cm}^{-1} \)), thus posing a limit on the sensitivity of the assay [92, 93]. Therefore, in section 4, a novel fluorogenic substrate for TL (Dabcyl-FKFLGKE-EDANS) is described, which was inspired by a previously documented dipeptide substrate (Dabcyl-SF-EDANS [94]), and TL’s specificity for the sequence Phe-Lys-Phe-Leu-Gly-Lys (P3 to P3’) [95]. The cleavage site, the kinetic parameters (\( K_M \) and \( k_{cat} \)), the effects of temperature and pH on the rate of peptide cleavage by TL, as well as the propensity of other proteases (e.g., serine, cysteine, and zinc proteases) to cleave the substrate will be determined. It is hypothesized that extending the length of an EDANS/Dabcyl-modified dipeptide substrate, and integrating TL’s specificity will increase both the substrate affinity and catalytic efficiency.
3. Unfolding and Metal Binding Studies on TL and CPA

3.1 Background

The pancreatic zinc-endopeptidase carboxypeptidase A (CPA) is one of the most studied zinc proteases. The divalent zinc cofactor is coordinated to Glu72, His69, His196, and a water molecule serving as the nucleophile in the peptide bond cleavage reaction (see Figure 1.3) [44]. In addition, CPA serves as a prototype for the M14 metallopeptidase family, and has been used extensively as a model protein to study the structure and catalytic mechanism of zinc enzymes [45]. Surprisingly, however, there is a lack of research regarding the influence of denaturants on the fold of the enzyme. Nevertheless, in 1962, Imohari and coworkers measured the optical rotation of CPA exposed to the chemical denaturing agent guanidine hydrochloride (GdnHCl) [96]. Their studies revealed the enzyme to unfold in the presence of 3 M GdnHCl with concomitant loss of enzymatic activity [96]. A few years later, Kronman and Holmes measured the tryptophan and tyrosine fluorescence of native and denatured (exposed to 6 M GdnHCl) CPA, revealing an increase in the tryptophan quantum yield from the native (0.128) to the denatured (0.147) state [97]. Over the last several decades, studies on CPA have been focused on its mechanism of action, rather than the structural changes accompanying (un)folding.

In contrast to CPA, many more structural aspects are known for thermolysin (TL). TL, the prototype of M4 family of metallopeptidases, is a calcium-binding zinc metalloendopeptidase isolated from the thermophilic organism Bacillus thermoproteolyticus [57-59]. Unlike CPA, TL harbours the metal ion binding motif HExxH...E. The catalytic zinc ion is coordinated to two histidine residues (His142 and His146), a glutamate residue (Glu166), and a water molecule (see
As previously shown in Figure 1.5, TL binds up to four calcium ions (numbered Ca1 – Ca4). The Ca$^{2+}$ ions do not participate in catalysis, but instead are found to stabilize the protein against thermal denaturation [70]. X-ray crystallographic analysis of TL suggested that there are no disulfide bonds in the enzyme, and that the calcium ions link together different parts of the protein, thus serving an important role in maintaining the integrity of TL’s native conformation and in increasing heat stability [98]. In fact, native TL is active at temperatures up to 80 °C [74], and optimally active between 60 – 70 °C [99-101]. It has been shown that the removal of calcium by chelators (such as EDTA) causes rapid autolytic degradation, presumably due to the partial unfolding of the enzyme [102]. In addition to the removal of the Ca$^{2+}$ ions, EDTA has also been shown to compete for the enzyme’s active site zinc ion. The removal of the catalytic zinc results in the loss of enzymatic activity [70]. This, however, is a reversible process. The catalytic activity can be restored upon the addition of zinc to the apo-enzyme, and the reconstitution of calcium results in restabilization of the enzyme and increased thermostability [70].

The carboxyl groups of Glu177, Asp185, and Glu190 coordinate the two closely bound Ca1 and Ca2 ions in the conserved double calcium binding site [103]. While Ca1-2 and Ca4 are located in the C-terminal domain (comprising residues 152 – 316), Ca3 is positioned in the N-terminal domain (residues 1 – 151) [72, 104]. The binding affinities of the four individual calcium sites remain controversial [72], however, there is a general agreement that the release of Ca3 or Ca4 results in the autolysis of thermolysin [71]. Site-directed mutagenesis studies have revealed that mutations in one surface-located region (residues 56-69), close to the Ca3 binding site, is involved in the unfolding of the enzyme, as well as in autolytic cleavage [105].
Similar to CPA, TL unfolds in the presence of the chemical denaturant GdnHCl. Corbett and coworkers have shown that the enzyme unfolds and irreversibly loses its enzymatic activity when exposed to 1.4 M GdnHCl [106]. Further investigations revealed TL to undergo autolytic degradation at this denaturant concentration [106]. This was confirmed using sedimentation and size exclusion chromatography-HPLC, where low molecular mass (< 5 kDa) fragments were observed upon autolysis, thus suggesting that the denaturant promotes the formation of a TL conformation susceptible to autolysis [106]. Therefore, it is crucial to eliminate autolysis when studying the unfolding of the enzyme exposed to GdnHCl.
3.2 Hypotheses and Objectives

The chromophoric chelator 4-(2-pyridylazo)resorcinol (PAR) has previously been employed as a probe to determine structural transitions during protein (un)folding in the zinc-dependent anthrax lethal factor protease (LF) [86]. In this study, it was found that LF unfolds in the presence of relatively low concentrations of denaturant (GdnHCl), and that the enzyme was capable of retaining its catalytic Zn$^{2+}$ ion even when unfolded [86]. An additional transition was observed when exposing the enzyme directly to PAR, which allows for the chelator to enter the enzyme’s active site. The study revealed that LF’s active site metal ion became accessible to chelation by PAR prior to its spontaneous release [86]. Therefore, the zinc protease is thought to transition from a PAR-inaccessible (closed) state to a PAR-accessible (open) form with increasing concentrations of GdnHCl.

The objective of this part of the thesis was to determine whether the transitions encountered with LF can be observed with all (or most) zinc proteases, and whether these structural transitions differ for each enzyme. For this work, thermolysin (TL) and carboxypeptidase A (CPA) were chosen, as they are amongst the best-studied zinc proteases. To achieve the aforementioned objective, the GdnHCl-induced unfolding, the spontaneous release of Zn$^{2+}$, and the accessibility of the active site metal ion to PAR, will be assessed for both TL and CPA in a comparative fashion. Hence, this study will determine whether PAR can be employed as a zinc accessibility probe (ZAP) to study structural transitions in zinc enzymes other than LF.

Prior to the unfolding studies, a suitable incubation period (of TL and CPA with GdnHCl) will be determined, since autolysis must be eliminated when studying the denaturant-induced unfolding of both zinc enzymes. SDS-PAGE analysis will be used to observe autolytic degradation.
Once a suitable incubation time is found, the unfolding of TL and CPA in the presence of GdnHCl will be monitored by tryptophan fluorescence spectroscopy. Furthermore, zinc release will be assessed following incubation of the enzyme with GdnHCl (at various concentrations), and subsequent Amicon filtration. Lastly, zinc accessibility will be determined by treating TL and CPA with GdnHCl prior to supplementation with PAR, and recording the immediate changes in absorbance.
3.3 Materials and Methods

3.3.1 Chemicals and laboratory equipment

All laboratory equipment and chemical reagents used throughout this work are listed in Tables 3.1 and 3.2, respectively.

Table 3.1: Laboratory equipment.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accumet™ AB150 pH meter with 6 mm glass pH electrode (calomel)</td>
<td>Fisher Scientific (Burlington, ON)</td>
</tr>
<tr>
<td>Amicon® Ultra Centrifugal Filters</td>
<td>Millipore Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Milli-Q Ultrapure water system</td>
<td>Fisher Scientific (Burlington, ON)</td>
</tr>
<tr>
<td>Cary 60 UV-Vis Spectrophotometer</td>
<td>Agilent (Santa Clara, CA)</td>
</tr>
<tr>
<td>Centrifuge 5415C</td>
<td>Eppendorf (Mississauga, ON)</td>
</tr>
<tr>
<td>Epoch Microplate spectrophotometer</td>
<td>BioTek Instruments (Winooski, VT)</td>
</tr>
<tr>
<td>Julabo CF31 water bath</td>
<td>Julabo (Seelbach, Germany)</td>
</tr>
<tr>
<td>Mini-PROTEAN Electrophoresis System</td>
<td>Bio-Rad Laboratory (Mississauga, ON)</td>
</tr>
<tr>
<td>OLIS RSM 1000 spectrophotometer/fluorometer</td>
<td>OLIS (Bogart, GA)</td>
</tr>
<tr>
<td>Pt100 probe</td>
<td>Julabo (Seelbach, Germany)</td>
</tr>
<tr>
<td>Xevo G2-XS Q-TOF mass spectrometer</td>
<td>Waters Corporation (Milford, MA)</td>
</tr>
<tr>
<td>Zeba Spin Desalting Column</td>
<td>Thermo Fisher Scientific (Burlington, ON)</td>
</tr>
<tr>
<td>Supplier</td>
<td>Reagent</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ACP Chemicals (Toronto, ON)</td>
<td>Silver nitrate</td>
</tr>
<tr>
<td>Bachem (Bubendorf, Switzerland)</td>
<td>$N$-(4-methoxyphenylazoformyl)-Phe-OH</td>
</tr>
<tr>
<td>BDH Chemicals (Poole, UK)</td>
<td>Citric acid</td>
</tr>
<tr>
<td>BioShop (Burlington, ON)</td>
<td>Acetic acid (glacial)</td>
</tr>
<tr>
<td></td>
<td>Acrylamide/Bis-Acrylamide (37.5:1; 30% solution)</td>
</tr>
<tr>
<td></td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td></td>
<td>Coomassie Brilliant Blue R-250</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
</tr>
<tr>
<td></td>
<td>Guanidine hydrochloride (ultra pure)</td>
</tr>
<tr>
<td></td>
<td>Protein Marker (0 kDa – 175 kDa)</td>
</tr>
<tr>
<td></td>
<td>$N$-2-Hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid (HEPES)</td>
</tr>
<tr>
<td></td>
<td>$N$-$N'$-$N'$-Tetramethylethlenediamine (TEMED)</td>
</tr>
<tr>
<td></td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride</td>
</tr>
<tr>
<td></td>
<td>Sodium dodecyl sulfate (SDS)</td>
</tr>
<tr>
<td></td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td></td>
<td>Tris(hydroxymethyl)aminomethane (Tris)</td>
</tr>
<tr>
<td></td>
<td>Triton X-100</td>
</tr>
<tr>
<td>Brenntag Canada Inc. (Calgary, AB)</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>Bio-Rad Laboratories (Hercules, CA)</td>
<td>Laemmli sample buffer</td>
</tr>
<tr>
<td>Fisher Scientific (Burlington, ON)</td>
<td>4-(2-Pyridylazo)resorcinol (PAR)</td>
</tr>
<tr>
<td></td>
<td>8-Anilinonaphthalene-1-sulfonic acid (ANS)</td>
</tr>
<tr>
<td></td>
<td>Calcium acetate</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
</tr>
<tr>
<td></td>
<td>Sodium thiosulfate</td>
</tr>
<tr>
<td></td>
<td>Zinc sulfate</td>
</tr>
<tr>
<td>Millipore Sigma (St. Louis, MO)</td>
<td>$\beta$-Mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>Calcium chloride</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde</td>
</tr>
</tbody>
</table>
3.3.2 Stock solutions

All aqueous solutions were prepared using Milli-Q ultrapure water (≥ 18.2 MΩ cm resistivity). HEPES buffer (50 mM, pH 7.4) was prepared by dissolving 11.9 g of HEPES in 1 L of water. The pH of buffer was adjusted using 1 M sodium hydroxide (NaOH). The HEPES buffer was kept at room temperature and was used for all experiments unless otherwise stated.

Stock solutions of Tris buffer (25 mM, pH 7.5) containing 0.5 M or 1 M NaCl were prepared by dissolving 7.6 mg of Tris, and 0.73 g (0.5 M) or 1.46 g (1 M) NaCl in 10 mL water. The pH of both buffers was adjusted using 1 M HCl, and they were kept at room temperature.

A 50 mL stock solution (8 M) of guanidine hydrochloride (GdnHCl) was prepared by dissolving 38.21 g of GdnHCl and 0.596 g of HEPES (50 mM final concentration) in water. The pH of the solution was adjusted to 7.4 using 1 M NaOH, and the stock solution was kept at room temperature.

A 10 mM stock solution of 4-(2-pyridylazo)resorcinol (PAR) was prepared by initially dissolving 108 mg of PAR in 5 mL of DMSO, followed by dilution to a final volume of 50 mL with HEPES buffer (50 mM, pH 7.4). An intermediate stock solution (1 mM) was also prepared by 10-fold dilution with HEPES buffer (50 mM, pH 7.4). Both stock solutions were covered in aluminum foil, and stored at 4 °C.

A 10 mM stock solution of the fluorescent probe 8-anilinonaphthalene-1-sulfonic acid (ANS) was prepared by dissolving 31.6 mg of ANS in 10 mL DMSO. The solution was covered in aluminum foil, and kept at 4 °C.
A 2.2 mM stock solution of the chromogenic substrate \(N-(4\text{-methoxyphenylazoformyl})-\text{Phe-OH}\) [107] was prepared by dissolving 8 mg of the peptide in 10 mL of DMSO. The stock solution was stored at -20 °C.

### 3.3.3 Enzyme preparation

Thermolysin (EC 3.4.24.27) from *Geobacillus stearothermophilus*, and the bovine pancreatic enzyme carboxypeptidase A (EC 3.4.17.1) were obtained from Millipore Sigma (St. Louis, MO), and were used without further purification.

Stock solutions of TL were prepared by dissolving 5 mg of the enzyme in 0.5 mL of HEPES buffer (50 mM, pH 7.4) containing 42% (v/v) glycerol, 0.001% (w/v) Triton X-100 and 10 mM CaCl\(_2\). The concentration of the enzyme was ascertained spectrophotometrically at 280 nm using an extinction coefficient of 58 220 M\(^{-1}\) cm\(^{-1}\) [108]. Stock solutions of CPA were prepared in Tris buffer (25 mM, pH 7.5) containing 1 M NaCl [109, 110]. The concentration of these protein solutions was determined using an extinction coefficient (\(\varepsilon_{280\text{ nm}}\)) of 64 200 M\(^{-1}\) cm\(^{-1}\) [109]. All enzyme stock solutions were stored at -80 °C.

### 3.3.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

A 13% polyacrylamide resolving gel and 8% polyacrylamide stacking gel was cast using the reagents shown in Table 3.3. A fresh Laemmli solution was prepared by mixing 300 µL of Laemmli sample buffer with 16 µL of \(\beta\)-mercaptoethanol. Protein samples (15 µM) were denatured by supplementation with an equal volume of Laemmli solution, followed by boiling for 5 min. Following denaturation, the samples were left on ice until they were loaded onto the gel. Running
buffer (composition shown in Table 3.4) was added to the Mini-PROTEAN Electrophoresis System. The samples (15 µL) were then loaded into the wells of the stacking gel, along with a Protein Marker (5 µL). Electrophoresis was performed initially at 75 V, and the voltage was increased to 150 V once the samples had migrated from the stacking gel into the resolving gel. Once the samples had passed through the gel, the resolving gel was retrieved, and stained with either Coomassie Blue or AgNO₃.

### Table 3.3: Composition of the polyacrylamide gels.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Resolving Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % Acrylamide/Bis-acrylamide</td>
<td>6.60 mL</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>3.75 mL</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris-HCl (pH 6.8)</td>
<td>-</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>10 % (w/v) Sodium dodecyl sulfate (SDS)</td>
<td>150 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>10 % (w/v) Ammonium persulfate (APS)</td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Water</td>
<td>4.66 mL</td>
<td>6.20 mL</td>
</tr>
</tbody>
</table>

### Table 3.4: Composition of the running buffer (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>72</td>
</tr>
<tr>
<td>Tris</td>
<td>15.1</td>
</tr>
<tr>
<td>SDS</td>
<td>5</td>
</tr>
</tbody>
</table>

#### 3.3.4.1 Coomassie Blue stain

Staining with Coomassie Blue was achieved by immersing the gel in the dye solution (composition listed in Table 3.5) for 5 min. The gel was then placed in destaining solution (see Table 3.6) for approximately 2 h, in order to visualize the bands. After destaining, the gel was stored in 10% (v/v) acetic acid at 4 °C.
Table 3.5: Composition of the Coomassie Blue staining solution (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Brilliant Blue R-250</td>
<td>2 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>455 mL</td>
</tr>
<tr>
<td>Water</td>
<td>452 mL</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>91 mL</td>
</tr>
</tbody>
</table>

Table 3.6: Composition of the destaining solution (1 L)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>300 mL</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>100 mL</td>
</tr>
<tr>
<td>Water</td>
<td>600 mL</td>
</tr>
</tbody>
</table>

3.3.4.2 Silver stain

One of the most sensitive methods for visualizing proteins separated by gel electrophoresis is silver staining [111]. The silver staining protocol adopted here was taken from the published literature [112]. Once the gel was retrieved from the electrophoresis system, it was immediately immersed in an aqueous fixing solution containing 40% (v/v) methanol and 5% (v/v) formaldehyde, for 10 min. The gel was then washed in water twice for 5 min each. After the wash, the gel was soaked in a 0.02% (w/v) sodium thiosulfate solution for 1 min, followed by washing in water twice for 20 s each. After the wash, the gel was immersed in a 0.1% (w/v) silver nitrate solution for 10 min. Afterwards, the gel was washed with water, and then again with dilute developing solution. The developing solution was prepared fresh, and consisted of 3% (w/v) sodium carbonate and 0.02% (v/v) formaldehyde in water. Once washed, the gel was soaked in the developing solution until band intensities were adequate. To terminate the development process, a solution of 2.3 M citric acid was added to the gel in the developing solution, and the
gel was placed on a shaker platform for 10 min. The gel was then washed in water for an additional 10 min, and then stored in 10% (v/v) acetic acid at 4 °C.

3.3.5 Tryptophan fluorescence spectroscopy

TL and CPA contain 3 and 7 tryptophan (Trp) residues, respectively [113, 114]. Thus, the unfolding of these zinc enzymes by chemical denaturants (such as GdnHCl) can be monitored by intrinsic Trp fluorescence spectroscopy using an excitation wavelength ($\lambda_{\text{exc}}$) of 295 nm. In a typical titration, TL or CPA (1 μM) was supplemented with GdnHCl (0 – 6 M) in HEPES buffer (50 mM, pH 7.4) for 10 min (for TL) or 1 h (for CPA) at room temperature prior to recording the emission spectra in a mirrored 10 × 4 mm quartz cuvette (Hellma, Concord, ON) using an OLIS RSM1000 spectrofluorometer (Bogart, GA) equipped with a 150 W xenon arc lamp, and a photon counter. The emission spectra of both TL and CPA were recorded from 300 to 400 nm in 1 nm increments (integration time of 1 s), with $\lambda_{\text{exc}}$ set to 295 nm. The excitation and emission bandpasses were 5 and 4 nm, respectively. All spectra were baseline-subtracted, and smoothed using the OLIS GlobalWorks software.

3.3.5.1 CPA

Exposure of CPA to GdnHCl for a period of 1 h was sufficient to reach the equilibrium of the unfolding transitions. The fluorescence intensities at 330, 340, and 350 nm ($F_{330}$, $F_{340}$, and $F_{350}$) from the recorded emission spectra, and their dependence on the concentration of denaturant was plotted, and fit by non-linear least-squares regression to a linear extrapolation
model represented by eq (1), describing a three-state mechanism (native (N) → intermediate (I) → unfolded (U)) with pre- and post-transitional baselines [115-117],

\[
F_{\text{obs}} = \frac{(y_N + s_N c) + F_{I} \times \exp\left(-\frac{\Delta G_{N \rightarrow I}^0 - m_N - s_N c}{RT}\right) + (y_U + s_U c) \times \exp\left(-\frac{\Delta G_{I \rightarrow U}^0 - m_I - s_U c}{RT}\right)}{1 + \exp\left(-\frac{\Delta G_{N \rightarrow I}^0 - m_N - s_N c}{RT}\right) + \exp\left(-\frac{\Delta G_{I \rightarrow U}^0 - m_I - s_U c}{RT}\right)}
\]

(1)

where \(F_{\text{obs}}\) is the observed fluorescence intensity at either 330 nm, 340 nm, or 350 nm, \(F_{I}\) represents the intensity of the intermediate state, \(y_N\) and \(y_U\) represent the intercepts, and \(s_N\) and \(s_U\) are the slopes of the pre- and post-transition baselines, respectively, \(\Delta G^0\) denotes the standard Gibbs free energy of a transition in the absence of denaturant, \(m\) is the corresponding-denaturant dependence of \(\Delta G^0\), \(c\) is the concentration of the denaturant, \(R\) is the gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)), and \(T\) is the temperature (293.15 K). The fit of the data to eq (1) was performed with the aid of GraFit 4.0 (Erithacus Software Ltd., Staines, UK) with \(\Delta G^0\), \(m\), \(s\), \(y\), and \(F_{I}\) serving as the fitting parameters.

In addition to the data analysis described above, global fits were performed with SigmaPlot 14.0 (Systat Software Inc., San Jose, CA) using eq (1). The global data set consisted of the emission intensities monitored between 330 and 350 nm (1 nm increments) as a function of the GdnHCl concentration. For each wavelength, values for the parameters \(y_N\), \(y_U\), \(s_N\), \(s_U\), and \(F_{I}\) were obtained, whereas the parameters \(\Delta G^0\) and \(m\) were global fit parameters, and common to all wavelengths.

The midpoint concentrations (\(C_{\text{mid}}\)) for the N → I and I → U transitions were calculated from the fitting parameters as shown in eq (2).
\[ C_{N \to l}^{mid} = \frac{\Delta G_{N \to l}^0}{m_{N \to l}} ; \quad C_{I \to U}^{mid} = \frac{\Delta G_{I \to U}^0}{m_{I \to U}} ; \quad C_{N \to U}^{mid} = \frac{\Delta G_{N \to U}^0}{m_{N \to U}} \]  

(2)

In addition, the thermodynamic parameters, \( \Delta G^0 \) and \( m \), obtained from the global fit of the data to eq (1) were used to determine the fractions of the native, intermediate, and unfolded states of CPA, with the aid of eq (3) to (5), respectively [118],

\[
f_N = \frac{\exp\left(-\frac{\Delta G_{N \to l}^0-m_{N \to l}c}{RT}\right)+\exp\left(-\frac{\Delta G_{I \to U}^0-m_{I \to U}c}{RT}\right)\times\exp\left(-\frac{\Delta G_{N \to U}^0-m_{N \to U}c}{RT}\right)}{1+\exp\left(-\frac{\Delta G_{N \to l}^0-m_{N \to l}c}{RT}\right)+\exp\left(-\frac{\Delta G_{I \to U}^0-m_{I \to U}c}{RT}\right)\times\exp\left(-\frac{\Delta G_{N \to U}^0-m_{N \to U}c}{RT}\right)}
\]  

(3)

\[
f_I = \frac{\exp\left(-\frac{\Delta G_{N \to l}^0-m_{N \to l}c}{RT}\right)}{1+\exp\left(-\frac{\Delta G_{N \to l}^0-m_{N \to l}c}{RT}\right)+\exp\left(-\frac{\Delta G_{I \to U}^0-m_{I \to U}c}{RT}\right)\times\exp\left(-\frac{\Delta G_{N \to U}^0-m_{N \to U}c}{RT}\right)}
\]  

(4)

\[
f_U = 1 - f_N - f_I
\]  

(5)

where \( f_N, f_I, \) and \( f_U \) represent the fraction of the native, intermediate, and unfolded states, \( \Delta G^0 \) denotes the standard Gibbs free energy of a transition in the absence of denaturant, \( m \) is the denaturant dependence of \( \Delta G^0 \), \( c \) is the concentration of denaturant, \( R \) is the gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)), and \( T \) is the temperature (293.15 K).

3.3.5.2 TL

For TL, the observed fluorescence intensities at 333 nm for TL (\( F_{333} \)) were plotted against the GdnHCl concentration in order to estimate the unfolding midpoint concentration (\( C_{\text{mid}} \)).
10 min incubation period with GdnHCl was insufficient for TL to reach equilibrium (see Results section). However, such short time of exposure to the denaturant was necessary to avoid substantial autolytic cleavage by the protease. Therefore, the data could not be fit by least-squares non-linear regression, and thus thermodynamic parameters ($\Delta G^0, m$) were not obtained. The $C_{mid}$ value of TL unfolding was estimated using the midpoint of the titration curve.

### 3.3.6 ANS fluorescence spectroscopy

The fluorescent molecular probe, 8-anilinonaphthalene-1-sulfonic acid (ANS), has been shown to possess a much stronger affinity for a molten globular (MG) state of a protein than for its native or unfolded state [119]. The protocol for the titration of CPA with GdnHCl in the presence of ANS was modified from the published literature [120-125]. CPA (2 µM) in HEPES buffer (50 mM, pH 7.4) was exposed to GdnHCl (0 – 4 M) for 1 h at room temperature prior to the addition of ANS (50 µM). The mixture was left to incubate for 5 min. The excitation wavelength ($\lambda_{exc}$) was set to 350 nm, and the emission was recorded from 400 to 600 nm in 1 nm increments (1 s integration time) with the aid of a 10 × 4 mm non-mirrored quartz cell, using the OLIS RSM1000 spectrofluorometer (Bogart, GA). Due to an inner filter effect (IFE), the recorded intensities (from 400 to 600 nm) were corrected by IFE correction factors (ranging from 1.633 to 1.535), which were determined based on the absorbance of ANS (50 µM) at both the excitation and emission wavelengths as outlined in the literature [126, 127].

The unfolding midpoints of CPA in the presence of ANS were determined by plotting $Fl_{480}$ against the GdnHCl concentration. The data was fit by least-squares non-linear regression to a linear extrapolation model represented in eq (6), describing a three-state mechanism of
unfolding (N → I → U), identical to that represented in eq (1) except for the omission of pre- and post-transitional baselines [115-117],

\[
FI_{obs} = \frac{FI_N + FI_I \times \exp \left( -\frac{\Delta G_{N \rightarrow I}^{0} - m_{N \rightarrow I} c}{RT} \right) + FI_U \times \exp \left( -\frac{\Delta G_{I \rightarrow U}^{0} - m_{I \rightarrow U} c}{RT} \right)}{1 + \exp \left( -\frac{\Delta G_{N \rightarrow I}^{0} - m_{N \rightarrow I} c}{RT} \right) + \exp \left( -\frac{\Delta G_{N \rightarrow I}^{0} - m_{N \rightarrow I} c}{RT} \right) + \exp \left( -\frac{\Delta G_{I \rightarrow U}^{0} - m_{I \rightarrow U} c}{RT} \right)}
\]  

where \( FI_{obs} \) is the observed fluorescence intensity at 480 nm, and all other parameters/constants are those outlined earlier. As described previously, data fitting was performed with the aid of GraFit 4.0 (Erithacus Software Ltd., Staines, UK) with \( \Delta G^0 \), \( m \), and \( FI_I \) serving as the fitting parameters.

Sigma Plot 14.0 (Systat Software Inc., San Jose, CA) was used to perform a global fit using eq (6). The global set consisted of all the emission intensities monitored between 420 and 520 nm (1 nm increments) as a function of the concentration of GdnHCl. The recorded fluorescence intensities at 420 – 520 nm were corrected by an IFE correction factor (ranging from 1.553 to 1.536), due to an inner filter effect caused by the presence of ANS. Unique values of the parameters \( FI_N \), \( FI_I \), and \( FI_U \) were obtained for each wavelength, whereas the parameters \( \Delta G^0 \) and \( m \) served as global fitting parameters and were common to all wavelengths.

The midpoint concentrations (\( C_{mid} \)) for the N → I and the I → U transitions were calculated by dividing \( \Delta G^0 \) by the corresponding \( m \) value (see eq (2)). The thermodynamic parameters \( \Delta G^0 \) and \( m \) obtained from the global fit were also used to determine the fractions of the N, I, and U states using eq (3), (4), and (5), respectively.
### 3.3.7 CPA activity assay

In a typical assay (total volume of 1 mL), CPA (50 nM) in HEPES buffer (50 mM, pH 7.4) was supplemented with 20 µM \(N\)-(4-Methoxyphenylazoformyl)-Phe-OH in a quartz cell. Immediately following the addition of the substrate, the change in absorbance at 350 nm was monitored for 60 s in 0.1 s intervals at 25 °C using a Cary 60 UV-Vis spectrophotometer (Agilent, Santa Clara, CA). The enzymatic activity was determined from the slope of the linear portion of the progress curve (0 – 15 s). The activity of CPA exposed to GdnHCl was measured in a manner analogous to that outlined above, except for a 1 h exposure of the enzyme to the denaturant prior to the addition of the substrate.

### 3.3.8 Reversibility of CPA unfolding

#### 3.3.8.1 Trp fluorescence in the absence of ANS

The \(U \to N\) transition was assessed by first exposing CPA (10 µM) to 5 M GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h. The guanidine-denatured sample was then diluted 10-fold with HEPES buffer (50 mM, pH 7.4) to mimic the native state (final concentration of 1 µM CPA in 0.5 M GdnHCl), prior to recording the emission spectra. A 10-fold dilution of CPA (10 µM) with 5 M GdnHCl served as a control (representing the unfolded state, with final concentration of 1 µM CPA in 5 M GdnHCl).

CPA (2 µM) was incubated in 5 M GdnHCl and HEPES buffer (50 mM, pH 7.4) for 1 h, in order to assess the \(U \to I\) reversibility. A 2-fold dilution with 5 M GdnHCl served as the unfolded state control sample (final concentration of 1 µM CPA in 5 M GdnHCl), whereas a 2-fold dilution with HEPES buffer (50 mM, pH 7.4) resulted in a final concentration of 1 µM CPA in 2.5 M GdnHCl.
(thus mimicking the intermediate state). The sample was diluted prior to recording the emission spectra.

The \( I \rightarrow N \) transition was evaluated by first exposing CPA (5 \( \mu \)M) to 2.5 M GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h. The stock was diluted 5-fold with 2.5 M GdnHCl (serving as the control (I state); final concentration of 1 \( \mu \)M CPA in 2.5 M GdnHCl) or HEPES buffer (final concentration of 1 \( \mu \)M CPA in 0.5 M GdnHCl; mimicking the N state) prior to recording the emission spectra. In addition, 2.5 M NaCl in HEPES buffer was used to dilute the initial stock of CPA 5-fold (final concentration of 1 \( \mu \)M CPA + 0.5 M GdnHCl + 2.0 M NaCl in HEPES buffer) in order to assess the effect of ionic strength on the \( I \rightarrow N \) transition.

All emission spectra were recorded in a mirrored 10 × 4 mm quartz cuvette (Hellma, Concord, ON) immediately upon dilution (and after defined intervals of time) using an OLIS RSM1000 spectrofluorometer (Bogart, GA). The excitation wavelength was set to 295 nm, and the emission spectra were recorded from 300 to 400 nm in 1 nm increments (integration time of 1 s). The excitation and emission bandpasses were 5 and 4 nm, respectively. All spectra were baseline-subtracted, and smoothed using the OLIS GlobalWorks software.

### 3.3.8.2 Trp fluorescence in the presence of ANS

The reversibility of CPA unfolding (\( U \rightarrow N \), \( U \rightarrow I \), and \( I \rightarrow N \) transitions) was assessed in a manner analogous to that outlined above, except for the supplementation of the samples with 50 \( \mu \)M ANS after diluting the initial CPA stock solutions with GdnHCl or HEPES buffer (see section 3.3.8.1). The emission spectra were recorded prior to the addition of ANS, and immediately after supplementation of ANS to the protein sample. The \( \lambda_{\text{exc}} \) was set to 295 nm, and the emission was
recorded from 300 to 600 nm in 1 nm increments (1 s integration time) using a 10 × 4 mm non-mirrored quartz cell, and the OLIS RSM1000 spectrofluorometer (Bogart, GA). The recorded intensities were corrected by IFE correction factors ranging from 1.671 to 1.488, which were determined based on the absorbance of ANS (50 μM) at both the excitation and emission wavelengths as outlined in the literature [126, 127]. The excitation and emission bandpasses were 5 and 4 nm, respectively. All spectra were baseline-subtracted, and smoothed using the OLIS GlobalWorks software.

3.3.8.3 ANS fluorescence

ANS-binding to CPA was measured during the renaturation of CPA (U → I, and I → N) by preparing the CPA stock solutions in a manner analogous to that outlined above (see section 3.3.8.1), except for the doubling of the CPA concentration (to obtain a final concentration of 2 μM), and supplementing the diluted enzyme solution with 50 μM ANS prior to recording the spectra. In this case, λexc was set to 350 nm, and the emission was recorded from 400 to 600 nm in 1 nm increments (1 s integration time) using a 10 × 4 mm non-mirrored quartz cell and the OLIS RSM1000 spectrofluorometer (Bogart, GA). As outlined earlier, the recorded intensities (from 400 to 600 nm) were corrected by IFE correction factors ranging from 1.633 to 1.535. The excitation and emission bandpasses were 5 and 4 nm, respectively. All spectra were baseline-subtracted, and smoothed using the OLIS GlobalWorks software.
3.3.9 Zinc release

The release of Zn\(^{2+}\) from TL and CPA was measured using a protocol taken from the published literature [86]. In a total volume of 0.5 mL, TL and CPA (5 \(\mu\)M each) in HEPES buffer (50 mM, pH 7.4) were exposed to GdnHCl (0 – 6 M) for 10 min (for TL) or 1 h (for CPA). After incubation, the samples were immediately subjected to filtration using a 0.5 mL Amicon Ultra Centrifugal Filter (30 kDa; Millipore Sigma, St. Louis, MO) at 14,000 \(\times\) \(g\) for 3 min in an Eppendorf 5415C Centrifuge (Eppendorf, Mississauga, ON). The concentration of released zinc was determined by transferring 190 \(\mu\)L of the filtrate to a 96-well microplate supplemented with 10 \(\mu\)L of 1 mM PAR (final concentration of 50 \(\mu\)M). The absorbance at 500 nm was recorded on an Epoch microplate reader. Zinc standards (0 – 10 \(\mu\)M) were measured in a similar manner, and served as the basis for the determination of the amount of Zn\(^{2+}\) released from TL and CPA at each GdnHCl concentration. The \(C_{\text{mid}}\) values of zinc release for both TL and CPA were estimated by plotting the amount of zinc released against the GdnHCl concentration, and determining the denaturant concentration associated to 50% zinc released. The 5 \(\mu\)M TL solution used in these investigations was found to contain 5.5 \(\mu\)M Zn\(^{2+}\), with 1 \(\mu\)M Zn\(^{2+}\) being free and unbound (determined following Amicon filtration and metal analysis of the filtrate by PAR). Therefore, the Zn\(^{2+}\) content for TL was found to be 0.9 Zn\(^{2+}\)/protein molecule. On the other hand, the Zn\(^{2+}\) content for CPA was determined to be 1.0 Zn\(^{2+}\)/protein molecule, with the 5 \(\mu\)M CPA solution containing 6 \(\mu\)M Zn\(^{2+}\) (total) and 1 \(\mu\)M free unbound Zn\(^{2+}\). Therefore, the amount of Zn\(^{2+}\) released was expressed in %, with 4.5 and 5 \(\mu\)M Zn\(^{2+}\) taken as 100%, for TL and CPA, respectively.
3.3.10 Zinc accessibility

The accessibility of TL- and CPA-bound zinc to chelation by PAR as a function of denaturant concentration was assessed in a manner similar to that described previously [86]. In a total volume of 200 µL, TL and CPA (5 µM each) were treated with GdnHCl (0 – 6 M) in HEPES buffer (50 mM, pH 7.4) for 10 min (for TL) or 1 h (for CPA) prior to supplementation with PAR (50 µM) in a 96-well microplate. The absorbance at 500 nm was recorded immediately using an Epoch microplate spectrophotometer (Biotek Instruments, Winooski, VT). Standards containing ZnSO₄ (0 – 10 µM), denaturant (at concentrations identical to those of the samples), and PAR (50 µM) were prepared and measured analogously along with the samples. The zinc standards served as the basis for the calculation of the amount of Zn²⁺ complexed by PAR. The zinc accessibilities (expressed in % with 4.5 and 5 µM Zn²⁺ bound to PAR taken as 100%, for TL and CPA, respectively) were plotted against the GdnHCl concentration, with 50% of zinc accessible to chelation by PAR representing the Cₘᵢₙ value.

3.3.11 Stopped-flow spectrophotometry

The kinetics of PAR-Zn²⁺ binding following exposure of CPA to PAR was monitored by stopped-flow UV-Vis spectrophotometry on an OLIS RSM1000 spectrophotometer (Bogart, GA) using a two-syringe setup with a 2 cm quartz cell. One syringe (2.5 mL) contained 75 µM PAR in 1.7 or 2.5 M GdnHCl in HEPES buffer (50 mM, pH 7.4), while the second syringe (0.5 mL) was loaded with Zn²⁺ standards (0 – 1 mM) or 6 µM CPA in 1.7 or 2.5 M GdnHCl in HEPES buffer (50 mM, pH 7.4). The enzyme was exposed to GdnHCl for 1 h prior to recording the absorbance spectra. Injection of the contents of the two syringes resulted in immediate mixing, and a final
concentration of 12.5 µM PAR (1/6 dilution), and a 5/6 dilution of the Zn\textsuperscript{2+} standards or the CPA solution (yielding a final concentration of 5 µM CPA). The spectra (ranging from 380 to 520 nm) were recorded for 3.5 s with a rate of 1000 scans/s immediately following injection. The spectra were baseline-subtracted with HEPES buffer (50 mM, pH 7.4) using the OLIS GlobalWorks software. To assess the degree of PAR-Zn\textsuperscript{2+} binding, the slopes of the linear portion of the progress curve (at 495 nm; 0 – 100 ms) were obtained for each Zn\textsuperscript{2+} standard. The standards served as the basis for the determination of the amount of accessible Zn\textsuperscript{2+} in CPA at each GdnHCl concentration.
3.4 Results

Changes upon lethal factor’s (LF) fold and metal-binding capacity upon exposure to chemical denaturants have previously been explored using a combination of chelator and tryptophan fluorescence spectroscopic studies [86]. The experiments described in this section were aimed at investigating the unfolding, zinc accessibility, and zinc release transitions for both thermolysin (TL) and carboxypeptidase A (CPA), with a view to determine whether the chelator PAR can be utilized as a zinc accessibility probe to investigate denaturant-induced structural transitions in zinc proteins other than LF.

3.4.1 Tryptophan fluorescence emission spectra of TL

Tryptophan (Trp) is the dominant intrinsic fluorophore in proteins, and its emission spectrum is widely used to study conformational transitions [126]. TL contains three Trp residues (Trp55, Trp115 and Trp186 [113]), and they can be selectively excited at a wavelength of 295 nm. As shown in Figure 3.1, the denaturation of TL (following exposure of the protein to 6 M GdnHCl and low Ca\textsuperscript{2+} concentration (50 µM) for 1 h) was accompanied by a redshift of the emission maximum (λ\text{max}) in the Trp emission spectrum from 333 to 350 nm. The redshift in λ\text{max} (from ~330 to 350 nm) upon denaturation has been previously documented in published literature [128], and suggests that the Trp residues in native TL are shielded from the solvent but become more accessible upon denaturation. As shown in Figure 3.1, the redshift was also accompanied by a 3.6-fold decrease in fluorescence intensity (at 333 nm). In fact, the enzyme was found to be completely denatured following exposure to 6 M GdnHCl (at low Ca\textsuperscript{2+} concentrations) for only 10 min, indicating a rapid transition from the native (N) to the unfolded (U) state under these
Supplementation of the medium with 10 mM Ca\(^{2+}\) appears to protect TL from unfolding as judged by the \(\lambda_{\text{max}}\) value remaining at 333 nm (indicative of the native state) after a 10 min and 1 h exposure to 6 M GdnHCl (see Figure 3.1). In addition, only moderate 1.5- and 1.7-fold decreases in the fluorescence intensities were observed at 333 nm after the 10 min and 1 h incubation periods, respectively (see Figure 3.1).

**Figure 3.1: Trp fluorescence spectra of native and GdnHCl-exposed TL.** TL (1 \(\mu\)M) was incubated in the absence (red trace), and presence of GdnHCl (6 M) at low ([Ca\(^{2+}\)] = 50 \(\mu\)M) and high ([Ca\(^{2+}\)] = 10 mM) calcium concentrations in HEPES buffer (50 mM, pH 7.4) for 10 min or 1 h prior to recording spectra. The excitation wavelength was set to 295 nm.
3.4.2 TL autolysis

Previous studies have revealed that TL undergoes autolytic degradation in the presence of GdnHCl [106, 128, 129]. Therefore, in this current investigation, initial experiments were aimed at finding conditions where complete denaturant-induced TL unfolding occurs prior to the onset of autolysis. As shown in Figure 3.2A, SDS-PAGE analysis revealed no signs of self-degradation of TL when the enzyme was exposed to 6 M GdnHCl in the presence of 10 mM Ca\(^{2+}\) for 1 h, but treatment with 6 M GdnHCl at low Ca\(^{2+}\) concentrations (50 µM) for 1 h revealed clear signs of protein autolysis. Hence, at low Ca\(^{2+}\) concentration, an incubation period of 1 h was deemed not suitable for studying TL unfolding.

As shown in Figure 3.1, TL was denatured when exposed to 6 M GdnHCl and low Ca\(^{2+}\) concentrations after 10 min only. Therefore, SDS-PAGE analysis of TL under these conditions was investigated, in order to observe signs of autolysis. As shown in Figure 3.2B, TL seems to be fully intact after a 10 min exposure to 6 M GdnHCl and 50 µM Ca\(^{2+}\). After the 10 min incubation, the enzyme was Amicon-filtered to separate the protein (retained in the retentate) from released Zn\(^{2+}\). As shown in Figure 3.2B, only slight self-degradation occurred when Amicon-filtered TL was left to incubate for 60 min, a finding not surprising since only 20% holo-TL remained in the retentate after Amicon filtration. However, substantial autolysis was observed after the addition of Zn\(^{2+}\) to the enzyme, suggesting that the metal ion is required for self-degradation of TL.
Figure 3.2: Silver stained SDS-PAGE gel of denatured and Amicon-filtered TL. (A) TL (10 µM) incubated with and without 6 M GdnHCl in HEPES buffer (50 mM, pH 7.4) at low (50 µM) or high (10 mM) Ca\(^{2+}\) concentrations for 1 h. (B) TL (10 µM) was exposed to GdnHCl (6 M) and 50 µM Ca\(^{2+}\) in HEPES buffer (50 mM, pH 7.4) for 10 min, and filtered through a 30 kDa Amicon ultrafiltration device. The retentate (containing 80% apoTL) was then allowed to incubate for 60 min in the absence or presence of zinc (10 µM) to assess its influence on autolysis.

A more detailed analysis of the time dependence of TL denaturation is shown in Figure 3.3. TL appears to be fully denatured following exposure to 6 M GdnHCl at low Ca\(^{2+}\) concentrations (50 µM) for 10 min. More importantly, SDS-PAGE analysis revealed most of TL to be intact under these conditions (see Figure 3.2B). In addition, TL exposed to 6 M GdnHCl at high Ca\(^{2+}\) concentrations (10 mM) for 1 h was not found to be fully denatured (see inset of Figure 3.3).
In fact, an exposure time of 24 h was required to reach the unfolded state upon exposure of the enzyme to 6 M GdnHCl with 10 mM Ca\textsuperscript{2+} (see Figure 3.3).

**Figure 3.3: Time dependence of TL unfolding.** TL (1 µM) was exposed to GdnHCl (6 M) at low (50 µM) or high (10 mM; depicted in the inset) Ca\textsuperscript{2+} concentrations in HEPES buffer (50 mM, pH 7.4) for the duration indicated.

TL autolysis (following exposure to 6 M GdnHCl and 10 mM Ca\textsuperscript{2+} for 1 – 24 h) was further investigated in hopes of finding a condition where complete denaturant-induced TL unfolding (at 10 mM Ca\textsuperscript{2+}) occurs prior to the onset of autolysis. As shown in Figure 3.4, SDS-PAGE analysis revealed the onset of TL autolysis to occur when the enzyme was incubated for 4 h. However, the inset of Figure 3.3 shows that, under these conditions, TL is not yet fully denatured until an
incubation period of 24 h. Therefore, it is impossible to study GdnHCl-induced TL unfolding in the presence of 10 mM Ca\(^{2+}\) due to the onset of autolysis prior to reaching the fully unfolded state. Thus, for the remainder of the studies, TL was exposed to GdnHCl with low Ca\(^{2+}\) for 10 min only, so as to avoid autolysis (rather than allowing TL unfolding to reach an equilibrium).

![Figure 3.4: SDS-PAGE/silver stain analysis on the time dependence of TL autolysis in the presence of 10 mM Ca\(^{2+}\). TL (10 \(\mu\)M) was exposed to GdnHCl (6 M) in HEPES buffer (50 mM, pH 7.4) with an additional 10 mM Ca\(^{2+}\) for the indicated time. An additional sample of TL (10 \(\mu\)M) exposed to GdnHCl for 1 h at low Ca\(^{2+}\) concentration (50 \(\mu\)M) served as a reference. HSA (final concentration of 0.25 mg/mL) served as a loading control.](image)

### 3.4.3 TL unfolding

The denaturation profile of TL exposed to GdnHCl (0 – 6 M) and 50 \(\mu\)M Ca\(^{2+}\) for 10 min is shown in Figure 3.5. The titration (assessed by monitoring the changes in the fluorescence intensities at 333 nm) revealed unfolding to proceed (most likely) via a two-state mechanism (N \(\rightarrow\) U) with an estimated midpoint concentration (\(C_{\text{mid}}\)) of 1.8 M. The \(C_{\text{mid}}\) value is defined as the concentration of denaturant at which both the folded and unfolded states are equally populated. Since a 10 min incubation period was not sufficient for the enzyme to reach
equilibrium, the data obtained from the titration (see Figure 3.5) cannot be fit by least-squares non-linear regression, and thus thermodynamic parameters could not be obtained.

**Figure 3.5: Unfolding profile for TL in the presence of GdnHCl.** TL (1 μM) was incubated with the indicated GdnHCl concentration and 50 μM Ca\(^{2+}\) in HEPES buffer (50 mM, pH 7.4) for 10 min prior to recording the fluorescence intensities at 333 nm. The vertical dashed line intersecting the titration curve denotes the estimated midpoint. The solid line connecting the data points does not indicate a fit of the data, but is meant to guide the eye. The excitation wavelength was set to 295 nm. Values shown represent the mean (± 1 s.d.) of three independent experiments.

### 3.4.4 Zinc accessibility and release from TL

Following the assessment of TL unfolding by Trp fluorescence spectroscopy, the ability of TL to retain its zinc ion, as well as the ability for PAR to access the metal in the active site (i.e., zinc accessibility) during GdnHCl-induced unfolding was investigated. As shown in Figure 3.6, 50% of Zn\(^{2+}\) was released (blue trace) at ~5 M GdnHCl, a concentration clearly beyond that required
for TL unfolding. Therefore, similarly to LF (see Figure 1.10), TL is capable of retaining its metal ion when unfolded. Analysis of the accessibility of TL’s Zn$^{2+}$ ion towards chelation by PAR revealed a virtually identical midpoint to that observed for zinc release (see Figure 3.6). Therefore, it appears that the structural changes required to make TL’s zinc ion accessible to chelation coincide with those triggering the release of the metal ion, an observation contrasting that reported for LF (see Figure 1.10).

**Figure 3.6: Influence of GdnHCl on the metal release (blue) and accessibility (red) of TL.** TL (5 µM) was exposed to the indicated concentrations of GdnHCl in HEPES buffer (50 mM, pH 7.4) for 10 min. The release of zinc was assessed following Amicon filtration, and analysis of the filtrate using PAR (50 µM). The metal accessibility was determined by treating the denaturant-exposed TL (5 µM) directly with 50 µM PAR (after the 10 min exposure time), and by immediately recording the absorbance at 500 nm. Values shown represent the mean (± 1 s.d.) of three independent experiments.
3.4.5 Tryptophan fluorescence studies on CPA

Similarly to TL and LF, a redshift in $\lambda_{\text{max}}$ (from 330 to 350 nm) was observed upon denaturation of CPA (exposed to 6 M GdnHCl for 1 h) (see Figure 3.7). As shown in Figure 3.8, Trp fluorescence spectroscopy revealed that CPA had reached equilibrium when exposed to GdnHCl for 1 h. In addition, SDS-PAGE analysis confirmed that, under these conditions, the enzyme does not undergo autolysis (data not shown). As shown in Figure 3.7, the $\lambda_{\text{max}}$ of 330 nm for native CPA suggests that the Trp residues are shielded from the solvent. Unfolding of CPA was accompanied by a 1.7-fold decrease in fluorescence intensity at 330 nm (see red and blue traces in Figure 3.7). However, at a moderate GdnHCl concentration (2.5 M), a redshift in $\lambda_{\text{max}}$ (from 330 to 340 nm) with concomitant increase in fluorescence intensity was observed, in which a 1.1- and 1.4-fold enhancement in the fluorescence intensity was observed at 330 and 340 nm, respectively. The enhancement in the emission of CPA at 2.5 M GdnHCl suggests an intermediate unfolding state (I). Enzymatic activity assays revealed that CPA exposed to 2.5 M GdnHCl for 1 h (i.e., representing the intermediate state) is completely inactive (showing < 1% activity). However, the enzyme was found to be 37% active when exposed to low concentrations of GdnHCl (0.5 M) for 1 h. Surprisingly, an intermediate state in CPA’s unfolding pathway has not been documented previously in the literature. Hence, the nature of the intermediate state was further investigated (see section 3.4.7).
Figure 3.7: Trp fluorescence spectra of CPA. CPA (1 μM) was incubated in the absence of GdnHCl (native state, red line), and presence of 2.5 M (intermediate state, green line) and 6 M (unfolded state, blue line) GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h prior to recording emission spectra. The excitation wavelength was set to 295 nm.

Figure 3.8: Time dependence of CPA unfolding. CPA (1 μM) was exposed to GdnHCl (6 M) in HEPES buffer (50 mM, pH 7.4) for the duration indicated. The excitation wavelength was set to 295 nm. The $F_{330\text{ nm}}$ value recorded at 120 min did not change from that determined at 60 min (data not shown).
3.4.6 CPA unfolding

The GdnHCl-mediated unfolding of CPA is shown in Figure 3.9. CPA unfolding appears to follow a three-state unfolding mechanism (N → I → U), as evidenced by the increase in Trp fluorescence intensity in the mid-concentration range. The N → I transition becomes more pronounced when monitoring the Trp fluorescence intensities at 340 nm (λ_{max} of the intermediate state; see Figure 3.9B) and 350 nm (λ_{max} of the unfolded state; see Figure 3.9C). As a result of CPA having three distinct states (N, I, and U), two midpoint concentrations (C_{mid}^{N→I} and C_{mid}^{I→U}) were determined. The unfolding profiles (recorded at 330, 340, and 350 nm; see Figure 3.9) were individually fit by least-squares non-linear regression to eq (1) (see section 3.3.5.1) describing a three-state mechanism with pre- and post-transitional baselines to obtain the thermodynamic parameters ΔG^{0} and m for the N → I and I → U transitions. The midpoint concentrations for both transitions (N → I and I → U) were then calculated from the thermodynamic parameters (see eq (2) in section 3.3.5.1).
Figure 3.9: Unfolding profile for CPA in the presence of GdnHCl. CPA (1 μM) was exposed to the indicated concentrations of GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h prior to recording the fluorescence intensities at (A) 330 nm, (B) 340 nm, and (C) 350 nm. The excitation wavelength was set to 295 nm. The solid line denotes a fit of the data according to a 3-state unfolding mechanism with pre- and post-transitional baselines (native → intermediate → unfolding) (see eq (1) in section 3.3.5.1). Values shown represent the mean (± 1 s.d.) of three independent experiments.
A summary of the parameters and midpoint concentrations for both transitions (N → I and I → U) from the individual wavelengths (330, 340, and 350 nm) for the unfolding of CPA is shown in Table 3.7. The $\Delta G_{N\rightarrow I}^0$ and $m_{N\rightarrow I}$ values (50.1 (± 24.4) kJ mol$^{-1}$ M$^{-1}$ and 23.6 (± 10.4) kJ mol$^{-1}$ M$^{-1}$, respectively) obtained from the fit using the data at 330 nm have high standard deviations, which likely arise from the shallowness of the N → I transition (see Figure 3.9A). The N → I transition in the unfolding profiles at both 340 and 350 nm (see Figure 3.9B and Figure 3.9C, respectively) is much more pronounced, therefore allowing for a more accurate fit of the data (as evidenced by the lower standard deviation). As a result, the calculated $C_{N\rightarrow I}^{mid}$ value (at 330 nm) differs slightly from those determined at 340 and 350 nm (see Table 3.7). On the other hand, values for both $\Delta G^0$ and $m$ for the I → U transition at each individual wavelength (330, 340 and 350 nm) were found to be very similar (~69 kJ mol$^{-1}$ and ~22 kJ mol$^{-1}$ M$^{-1}$, respectively). From the individual fits, the $C_{mid}$ values for the N → I and I → U transitions were determined to be around 2.0 M and 3.1 M, respectively.

Table 3.7: Summary of thermodynamic parameters and midpoints obtained from the individual and global fits of CPA unfolding.

<table>
<thead>
<tr>
<th>Thermodynamic Parameters and Midpoints</th>
<th>GdnHCl Titration$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>330 nm</td>
</tr>
<tr>
<td>$\Delta G_{N\rightarrow I}^0$ (kJ mol$^{-1}$)</td>
<td>50.1 (± 24.4)</td>
</tr>
<tr>
<td>$m_{N\rightarrow I}$ (kJ mol$^{-1}$ M$^{-1}$)</td>
<td>23.6 (± 10.4)</td>
</tr>
<tr>
<td>$C_{N\rightarrow I}^{mid}$ (M)</td>
<td>2.10 (± 0.09)</td>
</tr>
<tr>
<td>$\Delta G_{I\rightarrow U}^0$ (kJ mol$^{-1}$)</td>
<td>68.8 (± 14.2)</td>
</tr>
<tr>
<td>$m_{I\rightarrow U}$ (kJ mol$^{-1}$ M$^{-1}$)</td>
<td>22.1 (± 4.5)</td>
</tr>
<tr>
<td>$C_{I\rightarrow U}^{mid}$ (M)</td>
<td>3.11 (± 0.05)</td>
</tr>
</tbody>
</table>

$^a$ All values represent the mean (± 1 s.d.) of three independent experiments.
Although the $\Delta G^0$, $m$, and $C_{mid}$ values for the $N \rightarrow I$ and $I \rightarrow U$ transitions are similar at both 340 and 350 nm (see Table 3.7), it was desirable to determine the best fit of the thermodynamic parameters to a larger data set. To explore this further, the entire titration data set (with FI values assessed at each wavelength ranging from 330 to 350 nm) was globally fit to eq (1), in order to obtain one common $\Delta G^0$, $m$, and $C_{mid}$ value for each transition ($N \rightarrow I$ and $I \rightarrow U$) (see Figure 3.10). The parameters and midpoint concentrations (for both transitions) obtained from the global fit are summarized in Table 3.7 (last column). As Table 3.7 shows, in most cases, values of $\Delta G^0$, $m$, and $C_{mid}$ from the global fit are virtually identical to those obtained from the individual fits (at 330, 340, and 350 nm). In addition, the standard deviations associated to the fitting parameters and $C_{mid}$ values from the global fit are relatively similar to those determined at the individual wavelengths (except for the standard deviation of $\Delta G^0_N \rightarrow I$ and $m_{N \rightarrow I}$ at 330 nm). As shown in Table 3.7, the $C_{mid}$ values obtained from global fitting of CPA unfolding are very similar to the $C_{mid}$ values obtained from the individual fitting for both the $N \rightarrow I$ transition ($\sim 2.0$ M) and the $I \rightarrow U$ transition ($\sim 3.1$ M). Therefore, it can be concluded that both fitting techniques (individual and global fit) provided comparable $\Delta G^0$, $m$, and $C_{mid}$ values for each transition of CPA unfolding ($N \rightarrow I$ and $I \rightarrow U$).
Figure 3.10: Global fitting curves of GdnHCl-induced CPA unfolding. CPA (1 µM) was incubated at the indicated concentrations of GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h. The excitation wavelength was set to 295 nm, and the fluorescence intensities between 330 to 350 nm were monitored (1 nm increments). The data was globally fit according to a 3-state unfolding mechanism with pre- and post-transitional baselines (native → intermediate → unfolding), as shown by the dotted lines.

3.4.7 ANS fluorescence

The hydrophobic fluorescent probe, 8-anilinonaphthalene-1-sulfonic acid (ANS), was used to further characterize the intermediate state observed upon the denaturation of CPA. ANS has previously been shown to possess a strong affinity for the molten globular (MG) intermediate state of a protein [119]. In this state, the protein conserves a native-like secondary structure (partially or completely retained) accompanied by a distortion of the native three-dimensional structure [124]. ANS-binding is not observable in the protein’s native or unfolded states. The
hydrophobic core of a native protein is usually completely buried and protected from solvent, thus resulting in a low ANS-binding affinity [124]. As the protein unfolds (into the MG state), the hydrophobic regions become more exposed. This now allows the ANS molecules to bind noncovalently to the exposed hydrophobic patches of the protein (characterized by a large increase in fluorescence) [124]. In the unfolded state, the hydrophobic regions are completely exposed, and no ANS-binding is observed. The full exposure of the hydrophobic patches in the unfolded state results in a transfer of ANS molecules from a hydrophobic to a hydrophilic environment [125]. With ANS having a low fluorescence yield in polar environments [130], the transition from the MG intermediate state to the unfolded state is accompanied by a decrease in ANS fluorescence intensity.

The highest binding affinity for ANS is typically interpreted to arise from the presence of an MG intermediate state. ANS can be selectively excited at a wavelength of 350 nm [121, 123, 125]. As shown in Figure 3.11, the fluorescence intensity was increased 2.6-fold (at 480 nm) upon supplementation of CPA with 2.5 M GdnHCl for 1 h. In addition, the denaturation of CPA was accompanied by a blueshift of $\lambda_{\text{max}}$ in the ANS emission spectrum from 500 nm to 480 nm. The blue shift and the enhancement in the fluorescence intensity demonstrate that the probe is bound to newly exposed hydrophobic areas of CPA, thus suggesting the presence of an MG intermediate state at this concentration of GdnHCl (2.5 M). As shown in Figure 3.11, a low emission intensity was observed in the case of the native (red trace) and fully denatured (blue trace) CPA. The redshift of $\lambda_{\text{max}}$ (from 480 nm to 500 nm) observed upon the transition from the MG intermediate state to the unfolded state reflects a higher degree of accessibility for the
solvent to the hydrophobic regions (i.e., the ANS-binding region) within the protein (see Figure 3.11).

Figure 3.11: ANS fluorescence spectra of CPA exposed to GdnHCl. CPA (2 μM) was incubated with 0 M (native state, red line), 2.5 M (intermediate state, green line), and 4 M (unfolded state, blue line) GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h prior to supplementation with ANS (final concentration 50 μM), and incubation for an additional 5 min. Emission spectra were recorded using an excitation wavelength of 350 nm.

As shown in Figure 3.12, the ANS fluorescence in the presence of CPA was monitored at 480 nm using GdnHCl concentrations ranging from 0 to 4 M. The increase in ANS fluorescence at moderate concentrations of GdnHCl (2 – 3 M) suggests a three-state unfolding mechanism (N → I → U), an observation in agreement with the previously identified unfolding transitions using Trp
fluorescence (see Figure 3.9). The GdnHCl titration in the presence of ANS was fit to a three-state model (see eq (6) in section 3.3.6) to obtain the $\Delta G^0$ and $m$ values for each transition, thus allowing for the determination of the two midpoint concentrations ($C_{N\to I}^{\text{mid}}$ and $C_{I\to U}^{\text{mid}}$) (see eq (2) in section 3.3.5.1).

Figure 3.12: GdnHCl concentration dependence of the ANS fluorescence intensity during the unfolding of CPA. CPA (2 μM) was exposed to the indicated concentrations of GdnHCl for 1 h. Following incubation, ANS (final concentration 50 μM) was supplemented to the samples and left to incubate for 5 min, prior to recording the emission intensity at 480 nm. The excitation wavelength was set to 350 nm. All emission intensities were corrected by an IFE correction factor of 1.535. The data was fit to eq (6) (see section 3.3.6) describing a 3-state unfolding mechanism (native $\to$ intermediate $\to$ unfolding), as shown by the solid line. Values shown represent the mean ($\pm$ 1 s.d.) of three independent experiments.
In addition, the ANS fluorescence intensities at each wavelength (ranging from 420 to 520 nm in 1 nm increments) were globally fit to the same three-state model described above, thus yielding one value for $\Delta G^0$, $m$, and $C_{\text{mid}}$ for each transition. The fitting parameters and midpoint concentrations for both the individual and global fits are shown in Table 3.8. Similar to the values reported in Table 3.7, the fitting parameters and $C_{\text{mid}}$ values from the global fit are virtually identical to those obtained from the individual fit (at 480 nm). From the global fit, the $C_{\text{mid}}$ value for the N $\rightarrow$ I transition was found to be $\sim$1.7 M, whereas that of the I $\rightarrow$ U transition was $\sim$3.2 M (see Table 3.8).

Table 3.8: The thermodynamic parameters and midpoints derived from the individual and global fits of CPA denaturation in the presence of ANS.

<table>
<thead>
<tr>
<th>Thermodynamic Parameters and Midpoints</th>
<th>GdnHCl + ANS Titration$^a$</th>
<th>480 nm</th>
<th>Global Fit (420 – 520 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G_{N\rightarrow I}^0$ (kJ mol$^{-1}$)</td>
<td>15.0 (± 1.9)</td>
<td>16.1 (± 2.2)</td>
<td></td>
</tr>
<tr>
<td>$m_{N\rightarrow I}$ (kJ mol$^{-1}$ M$^{-1}$)</td>
<td>8.9 (± 1.1)</td>
<td>9.3 (± 1.4)</td>
<td></td>
</tr>
<tr>
<td>$C_{N\rightarrow I}^{\text{mid}}$ (M)</td>
<td>1.68 (± 0.03)</td>
<td>1.73 (± 0.06)</td>
<td></td>
</tr>
<tr>
<td>$\Delta G_{I\rightarrow U}^0$ (kJ mol$^{-1}$)</td>
<td>59.8 (± 12.5)</td>
<td>54.6 (± 11.1)</td>
<td></td>
</tr>
<tr>
<td>$m_{I\rightarrow U}$ (kJ mol$^{-1}$ M$^{-1}$)</td>
<td>18.6 (± 4.0)</td>
<td>16.9 (± 3.3)</td>
<td></td>
</tr>
<tr>
<td>$C_{I\rightarrow U}^{\text{mid}}$ (M)</td>
<td>3.22 (± 0.02)</td>
<td>3.23 (± 0.06)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ All values represent the mean (± 1 s.d.) of three independent experiments.

The thermodynamic parameters obtained from the global fit of both CPA unfolding titrations (with and without ANS) were used to determine the fraction of occupation of each state (N, I, and U). As shown in Figure 3.13, the transition from the native to the intermediate state differs, where a slightly lower midpoint concentration was observed in the GdnHCl-mediated CPA unfolding titration using ANS. However, the midpoint concentration for the I $\rightarrow$ U transition was found to be similar for both titrations. Furthermore, the maximum accumulation of the
An intermediate state occurred at 2.7 M GdnHCl ($f_i = 0.96$), regardless of how CPA unfolding was assessed (i.e., in the absence or presence of ANS) (see Figure 3.13).

**Figure 3.13:** Fractional occupancies of the native, intermediate, and unfolded states of CPA. The points of intersection represent the $C_{mid}$ values for each transition. The $C_{mid}$ values were determined from the thermodynamic parameters from global fit of the GdnHCl-mediated unfolding of CPA in the (A) absence and (B) presence of ANS.
3.4.8 Reversibility of CPA unfolding

The refolding of CPA was assessed by diluting the GdnHCl-denatured enzyme with appropriate amounts of denaturant and HEPES buffer, prior to analysis using intrinsic Trp and ANS fluorescence spectroscopy. As shown in Figure 3.14, the refolding of CPA from the U → N state was investigated. Rayleigh scattering (at 300 nm) was observed immediately upon the dilution of unfolded CPA, thus revealing the enzyme to misfold and aggregate (not visibly to the naked eye) under these conditions. In addition, the (instant) shift in $\lambda_{\text{max}}$ from 350 nm to 335 nm indicates that the enzyme does not completely refold into the native state, since a $\lambda_{\text{max}}$ of 330 nm is characteristic of the enzyme in the N state (see Figure 3.7). Even after a 2 h incubation, the $\lambda_{\text{max}}$ still remained at 335 nm, accompanied by a decrease in emission intensity (see Figure 3.14). This large quenching effect is likely due to the protein aggregates settling down in the cuvette, and thus not being directly exposed to the light source.

Although CPA did not fully refold into the N state (from the U state, see Figure 3.14), it was of interest to determine whether it was possible for the enzyme to refold into the intermediate state. Therefore, the U → I reversibility of CPA was assessed. As shown in Figure 3.15A, CPA was found to quickly refold to the intermediate state, where a shift in $\lambda_{\text{max}}$ from 350 nm to 340 nm was observed. Furthermore, the refolding of CPA from the U → I state did not induce Rayleigh scattering. Studies on the I → N reversibility on the other hand (shown in Figure 3.15B), revealed the enzyme to be incapable of completely refolding into the native state, and Rayleigh scattering was immediately observed upon dilution of the intermediate state with HEPES buffer (an observation in accordance with those documented for the U → N measurements). The I → N reversibility was also assessed by diluting CPA (exposed to 2.5 M
GdnHCl for 1 h, thus mimicking the I state) 5-fold with 2.5 M NaCl in HEPES buffer to maintain a constant ionic strength. Under these conditions, Rayleigh scattering was also observed immediately upon dilution, and similarly to the dilution with HEPES (in the absence of NaCl), the enzyme did not fully refold to the native state (i.e., $\lambda_{\text{max}}$ shifted from 340 to 335 nm) (see Figure 3.15B).

Figure 3.14: Trp fluorescence spectra of unfolded and partially renatured CPA. CPA (10 µM) was exposed to 5 M GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h. The CPA stock was diluted 10-fold with 5 M GdnHCl (to serve as the unfolded control; black line) or with HEPES buffer (red line) to mimic the conditions of the native state (final concentration of 1 µM CPA + 0.5 M GdnHCl in HEPES buffer). Emission spectra were measured at the indicated exposure times. The excitation wavelength was set to 295 nm.
Figure 3.15: Refolding of CPA from the unfolded and intermediate states. (A) CPA (2 μM) was incubated with 5 M GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h. The U → I reversibility was assessed by diluting the stock with 5 M GdnHCl (1:2 dilution, black line), to serve as a control for the unfolded state. The stock solution was also diluted with HEPES buffer (1:2 dilution, red line), prior to recording the emission spectra. The cyan line denotes a range of 5 to 60 min incubation of the HEPES-diluted CPA sample (final concentration of 1 μM CPA + 2.5 M GdnHCl). The excitation wavelength was set to 295 nm. (B) CPA (5 μM) was exposed to 2.5 M GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h. The CPA stock was diluted 5-fold with 2.5 M GdnHCl (to serve as the intermediate state control; black line) or with HEPES buffer (red line) to mimic the conditions of the native state (final concentration of 1 μM CPA + 0.5 M GdnHCl). The sample was left to incubate for 0.5 h (blue line), 1 h (cyan line), and 2 h (magenta line) prior to recording the emission spectra (λ<sub>exc</sub> = 295 nm). The black dashed line denotes a 5-fold dilution of the CPA stock solution with 2.5 M NaCl (to maintain a constant ionic strength) in HEPES buffer (50 mM, pH 7.4), and was incubated for 1 h prior to recording the emission spectrum (λ<sub>exc</sub> = 295 nm).
The reversibility of CPA’s 3-state unfolding mechanism was also assessed in the presence of ANS. As previously mentioned in section 3.4.7, ANS possesses a strong affinity for the molten globular (MG) intermediate state of a protein. A large increase in fluorescence is characteristic of the noncovalent binding of ANS to exposed hydrophobic patches of a protein [124]. As expected, the unfolded state control sample revealed a low emission intensity, with a $\lambda_{\text{max}}$ of 500 nm (see Figure 3.16A). The assessment of the reversibility of CPA from the $U \rightarrow I$ state revealed a blueshift of $\lambda_{\text{max}}$ (from 500 nm to 480 nm), and a 2.6-fold increase in $\text{FI}_{480\text{nm}}$ from the unfolded state. These observations were also recorded with the intermediate state control sample, thus further confirming the unfolded enzyme is capable of refolding (quickly) into the MG I state.

As previously shown in Figures 3.14 and 3.15B, the shift in $\lambda_{\text{max}}$ from 350 nm to 335 nm indicates that the enzyme does not completely refold into the native state (from the U and I states). The instant Rayleigh scattering observed upon the transition from the U or I states to the N state also indicate that CPA misfolds and aggregates under these conditions. Interestingly, as shown in Figure 3.16B, the misfolded enzyme shows a higher degree of exposed hydrophobic regions (i.e., the ANS-binding regions) within the protein, due to the 2.4-fold enhancement in fluorescence intensity at 480 nm. Therefore, these results suggest the aggregated and misfolded CPA contain more ANS-accessible hydrophobic patches than the protein’s MG state. From previous results, a 2.5-fold decrease in $\text{FI}_{480\text{nm}}$ would have been expected if the enzyme was refolding completely into its original native state. In addition, as shown previously in Figure 3.11, the GdnHCl-induced denaturation of CPA into the MG state (N $\rightarrow$ I) was accompanied by a blueshift of $\lambda_{\text{max}}$ in the ANS emission spectrum from 500 nm to 480 nm. Interestingly, in Figure 3.16B, an even greater blueshift of $\lambda_{\text{max}}$ (from 500 nm to 460 nm) was observed upon the
Figure 3.16: ANS fluorescence spectra of unfolded and partially renatured CPA. (A) The intermediate state control sample (blue line) was prepared by exposing CPA (2 μM) to 2.5 M GdnHCl for 1 h. Following incubation, ANS (final concentration 50 μM) was supplemented to the sample and left to incubate for 5 min, prior to recording the emission spectrum (λ<sub>exc</sub> = 350 nm). The unfolded state control (black line) represents CPA (2 μM) with 5 M GdnHCl (1 h incubation), and 50 μM ANS, which was left to incubate for 5 min. The U → I reversibility was assessed by first exposing CPA (4 μM) to 5 M GdnHCl for 1 h, and diluting the sample 2-fold with HEPES buffer (50 mM, pH 7.4) before supplementation with 50 μM ANS. The diluted CPA sample containing ANS (final concentration of 2 μM CPA + 2.5 M GdnHCl + 50 μM ANS) was left to incubate for 10 min, prior to recording the emission spectrum (red line). (B) The native state control (2 μM CPA + 0.5 M GdnHCl + 50 μM ANS) and the intermediate state control (2 μM CPA + 2.5 M GdnHCl + 50 μM ANS), are depicted in blue and black, respectively. The I → N reversibility was assessed by first exposing CPA (10 μM) to 2.5 M GdnHCl for 1 h, and diluting the sample 5-fold with HEPES buffer (50 mM, pH 7.4). The emission spectrum (λ<sub>exc</sub> = 350 nm) was measured immediately upon supplementation with 50 μM ANS (red line), after a 10 min incubation (green line), and after a 20 min incubation (cyan line).
renaturation of CPA from the intermediate state, thus indicating that the probe binds to more hydrophobic areas on the misfolded and aggregated form of CPA. Therefore, it can be concluded that CPA does not refold to a native or native-like state.

The fluorescent dye ANS has previously been used in Förster resonance energy transfer (FRET) studies to gain insight into the conformation of proteins [131]. In this FRET process, the Trp residues in the protein (i.e., the fluorescence donor) transfer energy to the quenching acceptor ANS, when in close proximity. The quenching of Trp fluorescence (following excitation at 295 nm) by ANS indicates that the ANS-binding sites are in the vicinity of Trp residues. Therefore, it was of interest to study the FRET interaction between Trp residues and the fluorescent probe ANS in CPA’s intermediate state and misfolded/aggregated states. As shown in Figure 3.17, the supplementation of ANS to the intermediate state control sample induced quenching of the intrinsic fluorescence of Trp ($\lambda_{\text{max}} = 340$ nm) with concomitant enhancement in ANS fluorescence ($\lambda_{\text{max}} = 460$ nm) after excitation at 295 nm. The quenching of Trp fluorescence accompanied by an increase in ANS emission is even more pronounced during the renaturation of CPA from the I state ($I \rightarrow N$ reversibility). In fact, the emission of ANS was found to exceed that of Trp, thus suggesting the existence of more hydrophobic cavities in the misfolded renatured-protein. As for the spectrum of the native state control sample, very little quenching of Trp fluorescence was observed (see Figure 3.17).
Figure 3.17: Trp fluorescence spectra of CPA assessing reversibility ($I \rightarrow N$) and quenching by ANS. The blue line denotes the native state control (1 µM CPA + 50 µM ANS in HEPES buffer (pH 7.4)) whereas the magenta and black lines represent the intermediate state control (1 µM CPA exposed to 2.5 M GdnHCl in HEPES buffer) in the absence and presence of 50 µM ANS, respectively. CPA (5 µM) was exposed to 2.5 M GdnHCl in HEPES buffer for 1 h, and was diluted 5-fold with HEPES buffer (final concentration 1 µM CPA + 0.5 M GdnHCl), thus mimicking the conditions of the native state. Following dilution, ANS (final concentration 50 µM) was added to the sample, and left to incubate for 1 h, prior to recording the emission spectrum (red line). The excitation wavelength was set to 295 nm.

3.4.9 Zinc accessibility and release from CPA

The ability of CPA to retain its zinc ion, as well as the ability of PAR to chelate the catalytic metal ion during GdnHCl-mediated unfolding, was assessed in a fashion similar to that reported for TL and LF (see section 3.4.4). As shown in Figure 3.18, the zinc accessibility and release transitions were found to differ, an observation documented for LF, but not for TL (see Figures 1.10 and 3.6). Analysis of the zinc accessibility by PAR revealed that 50% of CPA’s metal ion was accessible to chelation by PAR at ~1.9 M GdnHCl. Furthermore, 50% of zinc was released at 3.4 M GdnHCl, a concentration clearly beyond the $C_{\text{mid}}$ value determined for the zinc accessibility...
(see Figure 3.18). Interestingly, the zinc accessibility midpoint concentration appears to coincide nicely with the $N \rightarrow I$ transition midpoints values, where the $C_{\text{mid}}$ values for the $N \rightarrow I$ transition in the absence and presence of ANS were 2.0 M and 1.7 M, respectively. In addition, GdnHCl concentrations of 3.2 M and 3.1 M represent the $C_{\text{mid}}$ values for the $I \rightarrow U$ transition with and without ANS, respectively. The $C_{\text{mid}}^{\text{I} \rightarrow \text{U}}$ value of ~3.2 M thus only slightly differs from the zinc release $C_{\text{mid}}$ value of 3.4 M. It is plausible that this difference can be attributed to the relatively large error bars associated with the data points in the transitional region of the S-curve shown in Figure 3.18.

![Graph](image)

**Figure 3.18: Influence of GdnHCl on the metal release (blue) and accessibility (red) of CPA.** CPA (5 µM) was exposed to the indicated concentrations of GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h. The release of zinc was assessed following Amicon filtration, and analysis of the filtrate using PAR (50 µM). The metal accessibility was determined by treating the denaturant-exposed CPA (5 µM) directly with PAR (50 µM), and by immediately recording the absorbance at 500 nm. Values shown represent the mean (± 1 s.d.) of three independent experiments.
3.4.10 Stopped-flow spectrophotometry of PAR-bound Zn\textsuperscript{2+} in CPA

The rate of the chelation of Zn\textsuperscript{2+} by PAR was investigated with a view to determine whether the initial kinetics of this reaction differ between protein-bound Zn\textsuperscript{2+} and that of the free metal ion in solution. Differences in the kinetic profiles would imply that the reaction of PAR with CPA’s zinc ion at intermediate GdnHCl concentrations (where an open, PAR-accessible conformation is postulated) proceeds via a path distinct from that encountered with the chelation of the free (dissociated) metal ion in solution. In other words, such differences would suggest that at intermediate GdnHCl concentrations, the Zn\textsuperscript{2+} ion is indeed bound to CPA, and not loosely attached or free in solution.

In order to probe the initial kinetics of the capture of Zn\textsuperscript{2+} from CPA in the intermediate state, two concentrations of GdnHCl were chosen: 1.7 M (which represents the midpoint concentration of the N → I transition determined in section 3.4.7) and 2.5 M (which is close to the concentration at which the maximum occupancy of the I state was observed). At the onset of these investigations, the increases in absorbance at 495 nm were measured in 1 ms intervals for samples containing PAR and different concentrations of (free) Zn\textsuperscript{2+} (i.e., in the absence of CPA). As Figure 3.19 shows, the reaction of PAR with free Zn\textsuperscript{2+} is rapid, with completion of this process achieved after a few seconds. In addition, the progress of the reaction during the first 100 ms was found to be linear for all Zn\textsuperscript{2+} concentrations (except under saturating conditions using 833 µM Zn\textsuperscript{2+}, where the reaction was too fast). Furthermore, the initial velocities (slopes) recorded within the first 100 ms were found to correlate linearly with the Zn\textsuperscript{2+} concentration (for 1.7 M GdnHCl: slope = 0.2776 × [Zn] + 0.2895, R\textsuperscript{2} = 0.997; for 2.5 M GdnHCl: slope = 0.2201 × [Zn] + 0.2019, R\textsuperscript{2} = 0.998). As shown in Figure 3.19A, the progress curve recorded for CPA at a
GdnHCl concentration of 1.7 M is much shallower, and therefore differs significantly from those obtained with free Zn$^{2+}$. This result clearly suggests that most of the metal ion in the CPA-containing sample is not free (i.e., it must be protein-bound), and that its chelation is overall a slower process. Based on an analysis of the velocity (slope = 0.511) during the first 100 ms (using the linear correlation stated above), PAR was found to bind to 0.8 µM Zn$^{2+}$ rapidly. This finding is consistent with the previously determined concentration of free Zn$^{2+}$ in 5 µM CPA solutions (which contain 6 µM Zn$^{2+}$ in total, with 1 µM being free). Hence, the initial phase of the reaction is determined by the capture of free Zn$^{2+}$ in the CPA solution, whereas the shallow increase that follows can be attributed to the much slower reaction of PAR with the protein-bound metal ion. Similar observations were recorded for CPA exposed to 2.5 M GdnHCl (see Figure 3.19B), where analysis of the first 100 ms of the progress curve revealed a concentration of 1.7 µM (readily sequestered) Zn$^{2+}$, and a shallow increase in metal complexation beyond that time. It is interesting to note that the increase from 0.8 to 1.7 µM free Zn$^{2+}$ upon increasing the concentration of the denaturant is in general agreement with the metal release studies (see Figure 3.18), where an onset of metal dissociation from CPA was observed at GdnHCl concentrations above 2 M. Overall, the stopped-flow spectrophotometric data indicate that the kinetics of the reaction of PAR with CPA are different from those recorded for free Zn$^{2+}$, an observation that supports the notion that, at intermediate denaturant concentrations, PAR is not abstracting free or loosely-bound metal, but rather interacts directly with the enzyme (presumably via the formation of a CPA:Zn:PAR ternary complex).
Figure 3.19: Stopped-flow UV-Vis spectrophotometric determination of the progress of Zn$^{2+}$ chelation by PAR. CPA (6 µM) was exposed to 1.7 M or 2.5 M GdnHCl in HEPES buffer (50 mM, pH 7.4) for 1 h prior to initiation of the reaction with PAR. A 2.5 mL syringe was loaded with 75 µM PAR (using the appropriate GdnHCl concentration), whereas a 0.5 mL syringe was filled with either the CPA sample or Zn$^{2+}$ standards (in GdnHCl). Rapid mixing of the contents of the syringes resulted in final concentrations of 12.5 and 5.0 µM PAR and CPA, respectively. The kinetic traces for CPA are shown in black. The final concentrations of the Zn$^{2+}$ standards were as follows: 0 µM (red), 1.00 µM (orange), 1.67 µM (yellow), 2.50 µM (green), 3.33 µM (cyan), 6.67 µM (blue), and 833 µM (purple). The absorbance at 495 nm was monitored in 1 ms intervals for a total of 3.7 s. Panels A and B show the progress curves determined with 1.7 M and 2.5 M GdnHCl, respectively.
3.5 Discussion

The chromophoric chelator PAR has been utilized previously as a chelating agent in spectrophotometric determinations of Zn\(^{2+}\) in biological materials [89]. As such, it has been used to measure the total Zn\(^{2+}\) content in proteins, as well as the concentration of the metal ion following its release from denatured proteins [132-135]. More recently, PAR has been employed to study the fold and metal dissociation in the zinc-dependent LF [86]. This study revealed LF to unfold in the presence of relatively low concentrations of the denaturant GdnHCl, and that the enzyme was capable of retaining its catalytic zinc ion even when unfolded. In addition, a new transition was observed when exposing LF directly to PAR, revealing the chelator to gain access to the active site metal ion prior to its spontaneous release. Whether such transitions occur in TL and CPA, and whether PAR can therefore serve as a general zinc accessibility probe (ZAP) to study protein (un)folding in zinc enzymes, was investigated in this chapter.

3.5.1 TL unfolding and autolysis

It has previously been shown that TL undergoes autolytic degradation in the presence of chemical denaturants, including GdnHCl [106]. Therefore, initial experiments were aimed at finding conditions where complete GdnHCl-induced TL unfolding occurs prior to the onset of autolysis. In previous studies using GdnHCl, Khan and coworkers allowed TL samples to equilibrate for 24 h before measurements were taken, whereas, Fontana and coworkers measured the fluorescence of TL after 5 min of exposure to the denaturant [128, 136]. Corbett and coworkers argued that the enzyme undergoes extensive autolysis within 3 to 4 h, and that unfolding in the range of 0 – 2 M GdnHCl requires a duration longer than 5 min. Therefore, the
authors allowed for a 1 h incubation period rather than 24 h (i.e., equilibrium measurements) or 5 min [106]. However, in the preliminary unfolding and autolysis studies described in this work, it was found that TL unfolds rapidly (within 1 min) when exposed to 6 M GdnHCl at low concentrations of Ca\(^{2+}\) (50 µM) (see Figure 3.3). In contrast, in the presence of 10 mM Ca\(^{2+}\), 24 h was required to fully unfold the enzyme. SDS-PAGE analysis revealed the enzyme was still intact when exposed to GdnHCl (6 M) at low Ca\(^{2+}\) concentrations for 10 min, but had self-degraded after 1 h under these conditions. In addition, the onset of TL autolysis occurred when the enzyme was incubated with 6 M GdnHCl at high Ca\(^{2+}\) concentrations for 4 h. Therefore, it is impossible to study GdnHCl-induced TL unfolding in the presence of 10 mM Ca\(^{2+}\), due to the onset of autolysis prior to reaching the unfolded state. As a consequence, it was deemed more important to study the unfolding of TL under conditions that minimize autolysis (i.e., TL exposed to 6 M GdnHCl + 50 µM Ca\(^{2+}\) for 10 min), rather than allowing the enzyme to reach equilibrium.

The denaturation profile of TL exposed to GdnHCl at low concentrations of Ca\(^{2+}\) revealed a C\(_{\text{mid}}\) value of 1.8 M (see Figure 3.5). In addition, 50% of the enzyme’s catalytic zinc was released at ~5 M GdnHCl, a concentration clearly beyond that required for TL unfolding. These results suggest that the protease is capable of retaining its metal ion even when unfolded (an observation similar to that reported for LF). Interestingly, exposing TL to the chelator PAR following incubation with the denaturant did not reveal an additional transition. In fact, the midpoint concentration was virtually identical to the C\(_{\text{mid}}\) value of the Zn\(^{2+}\) release transition (see Figure 3.6). Therefore, a distinct accessibility transition does not exist for TL. This observation contrasts that documented for LF, where it was proposed that PAR gains access to LF’s active site zinc ion once in a denaturant-induced accessible (open) state, and that PAR chelates the zinc ion
prior to its spontaneous release. The lack of a distinct accessibility transition in TL therefore indicates that PAR does not gain access to the protein-bound zinc ion before its release from the active site. As such, there appears to be no open PAR-accessible state in the unfolding mechanism of TL.

### 3.5.2 CPA unfolding

An MG intermediate state was observed in CPA’s (GdnHCl-induced) unfolding pathway, as evidenced by the redshift in $\lambda_{\text{max}}$ from 330 to 340 nm, and an enhancement in fluorescence intensity. This increase in FI observed in the Trp emission spectrum is similar to that found with other proteins for which an MG state has been elucidated, including carbonic anhydrase II [137], glucoamylase [138], and $\alpha$-fetoprotein [139]. Surprisingly, an I state in CPA’s unfolding pathway has not been documented previously in the literature. However, carboxypeptidase Y (CPY), a vacuolar serine protease from *Saccharomyces cerevisiae* [140], was found to unfold through a multistep process involving at least three transition states, with two transitions leading to the formation of a molten globule-like state [123, 141]. Denaturation of CPY was induced by high pressure, and the studies revealed that an increase in pressure results in the onset of ANS-binding accompanied with the inactivation of the enzyme (two characteristic properties of an MG structure), with the latter being completely irreversible at pressure values above 300 MPa (or incubation at 200 MPa for 24 h) [141]. The increase in ANS-binding was observed at values higher than 250 MPa, thus indicating that CPY’s surface developed a more hydrophobic character (a feature characteristic of a protein’s MG state) at this pressure. An increase in ANS fluorescence (at the I state) was also observed in the studies reported in this work. This enhancement of ANS
emission demonstrates that the probe is binding to newly exposed hydrophobic areas of CPA upon denaturation, thus suggesting the presence of an MG intermediate state. In the current studies, however, unfolding of CPA was induced with GdnHCl, rather than by increases in pressure (known as a reagentless denaturant). Furthermore, CPY is proposed to follow the denaturation pathway $N \rightleftharpoons N' \rightleftharpoons MG_1 \rightleftharpoons MG_2$, with two sequential intermediates exhibiting MG structure, and the final state (MG$_2$) showing enhanced ANS-binding [123]. In fact, multiple MG state transitions have previously been reported for other proteins, including horse cytochrome c and hen egg white lysozyme [142, 143]. The proposed unfolding pathway for CPY differs from the one proposed here for CPA ($N \rightarrow MG \rightleftharpoons U$), where the protein converts directly from the MG intermediate state to the completely denatured state, as evidenced by the redshift in $\lambda_{\text{max}}$ (from 340 to 350 nm), and the 1.7-fold decrease in fluorescence intensity (at 330 nm) upon exposure of CPA to 6 M GdnHCl for 1 h (see Figure 3.7). The redshift and the quenching of $\text{Fl}_{330 \text{ nm}}$ suggest that the Trp residues in CPA are exposed to a more polar environment, which is characteristic of a protein’s unfolded state.

3.5.3 Reversibility of CPA unfolding

The reversibility of the structural changes in CPA was investigated in the absence and presence of ANS, using both intrinsic Trp and ANS fluorescence spectroscopy. These studies revealed CPA to quickly refold into the MG intermediate state from the unfolded state. On the other hand, Rayleigh scattering was observed immediately upon the dilution of CPA from both the U and I states, to the N state, suggesting that the enzyme aggregates under these conditions. In addition to the scattering, the $\lambda_{\text{max}}$ remained at 335 nm, and did not reach 330 nm (the $\lambda_{\text{max}}$
characteristic of native CPA). Furthermore, the studies on the reversibility of the $N \rightarrow I$ transition in the presence ANS revealed that the misfolded/aggregated enzyme possesses a higher degree of exposed hydrophobic regions within the enzyme (in comparison to the MG I state), as evidenced by the large enhancement in the emission at 480 nm, and the blueshift of $\lambda_{\text{max}}$ from 480 nm to 460 nm. Taken together, these results suggest that CPA unfolds via the following three-state unfolding pathway: $N \rightarrow MG \rightleftharpoons U$.

It is interesting to point out that in the case of CPY, the observed conformational changes were also only partly reversible [123, 141]. For instance, after the release of pressure (from 300 MPa), the original, native $\lambda_{\text{max}}$ and peak intensity in the enzyme’s Trp emission spectrum were not completely restored, indicating that the change in CPY’s conformation induced by high pressure is only partly reversible [141]. In addition, pressure release from the highest pressure tested (600 MPa) did not result in an enhancement in ANS emission at 475 nm, but rather a decrease in FI$_{475 \text{ nm}}$ (however not completely restored to the FI$_{475 \text{ nm}}$ at 0 MPa), an observation contrasting that reported for CPA in the studies described in this chapter (see Figure 3.16B). The authors propose that the irreversibility of the transition(s) in CPY may be due to the possible aggregation of the (partially) unfolded protein, or due to a pro-region in the pre-matured protein being required for the correct (re)folding of CPY [123, 141, 144]. As for CPA, the pro-region is known to inhibit the enzyme with a $K_i$ of $10^{-10}$ M [145]. In addition, the proteolytic cleavage of pro-CPA (403 residues) by trypsin results in the separated enzyme and activated segment moieties [146]. However, the pro-region’s role in folding has not yet been examined [147]. Since the pro-region of CPY is required for the correct folding of the precursor, it is possible that this is
also true for CPA, and thus would explain why the protease cannot refold completely to the native state.

As previously mentioned, CA II was found to form an MG intermediate structure [137]. The kinetics of refolding of CA II have been investigated by various methods (including ANS-binding), and these studies have revealed the enzyme to refold reversibly in three stages, with the native active protein being formed in the last stage [148]. Refolding from the MG state to the native state of CA II was also investigated by adding Zn$^{2+}$ or Co$^{2+}$ to the intermediate state of the apoenzyme, to explore the role of the metal ion and its effects on the folding reaction [149]. These studies demonstrated that Zn$^{2+}$ stabilizes the MG state, and that, at 1.2 M GdnHCl, refolding was possible by simply adding the metal cofactor to the MG state of the apoenzyme instead of changing the denaturant concentration [149]. Refolding of CPA by reconstituting the MG or unfolded apo-enzyme with Zn$^{2+}$ was not assessed in this thesis. Indeed, it would be of interest to determine if the outcome is similar to that reported for CA II.

In addition, the scrapie amyloid (prion) protein, a component of the infectious scrapie agent, was found to contain molten globule-like characteristics in the protein’s intermediates [120]. The refolding of the unfolded scrapie amyloid (prion) protein was unsuccessful due to the formation of misfolded aggregates, a finding similar to that observed in the current studies on CPA.

### 3.5.4 Solvent-accessible surface area of CPA

The denaturant-dependence of the Gibbs free energy of unfolding ($m$ value) has been found to correlate with changes of the solvent-accessible surface area ($\Delta$SASA) of a protein [150].
The $\Delta$SASA (expressed in Å$^2$) value can be estimated fairly accurately based simply on the number of a protein’s amino acid residues (eq (7) [150]),

$$\Delta\text{SASA} = -907 + 93 (\text{res#})$$  \hspace{1cm} (7)

where $\text{res#}$ represents the number of amino acid residues in a protein. With CPA having 307 residues [48, 49], a $\Delta$SASA value of 27 644 Å$^2$ was determined. The value of $m$ (for GdnHCl-induced denaturation) can be calculated using eq (8) [150],

$$m = (859 + 0.22(\Delta\text{SASA})) \left( \frac{4.184 \times 10^{-3} \text{kJ}}{1 \text{cal}} \right)$$  \hspace{1cm} (8)

where $m$ and $\Delta$SASA are expressed in kJ mol$^{-1}$ M$^{-1}$ and Å$^2$, respectively. Using the $\Delta$SASA value of 27 644 Å$^2$ therefore leads to an $m$ value of 29.0 kJ mol$^{-1}$ M$^{-1}$ for the unfolding of CPA (N $\rightarrow$ U). This value coincides reasonably well with the $m_{N\rightarrow U}$ values ($m_{N\rightarrow I} + m_{I\rightarrow U}$) obtained from the global fits of CPA unfolding in the absence (34.8 kJ mol$^{-1}$ M$^{-1}$) and presence (26.2 kJ mol$^{-1}$ M$^{-1}$) of ANS (see Tables 3.7 and 3.8, respectively). In addition to the calculations using equations (7) and (8), the $\Delta$SASA value was determined from the SASA values of the native (SASA$_N$) and fully unfolded (SASA$_U$) states of CPA. SASA$_N$ of a protein can be obtained using online tools such as GetArea [151] and FreeSASA [152], provided that its crystal structure is known. The SASA$_N$ value for native CPA (PDB ID: 5CPA [50]) was found to be $\sim$12 200 Å$^2$ using both online tools. The assessment of SASA$_U$ is less trivial, and there are a variety of methods to estimate its value. Lu and Wagaman have compared eight different methods of computing SASA$_U$, and, in the end, the authors
advocate the use of the (lower-bound) Gong/Rose method [153, 154]. Using this method, the SASA\textsubscript{U} value for CPA was calculated to be \(\sim 42\,600\,\text{Å}^2\). Thus, the overall change of the SASA from the folded to the unfolded state of CPA is estimated to be 30 400 Å\(^2\) (i.e., 42 600 – 12 200 Å\(^2\)), a value similar to that determined using equations (7) and (8). Substituting the \(\Delta\text{SASA}\) value determined above into eq. (8) yields an \(m_{N\to U}\) value of 31.6 kJ mol\(^{-1}\) M\(^{-1}\), which is in between those obtained from the global fits of the titrations performed in the absence and presence of ANS (34.8 and 26.2 kJ mol\(^{-1}\) M\(^{-1}\), respectively). As such, the experimentally observed \(m_{N\to U}\) values for CPA appear to be in general agreement with those expected based on semi-empirical and theoretical (Gong/Rose) methods.

3.5.5 Summary of unfolding, zinc accessibility and release studies

The GdnHCl-induced protein unfolding, the spontaneous release of the metal ion, and the accessibility of the active site Zn\(^{2+}\) ion by PAR were investigated for both TL and CPA in this study, in a manner similar to that reported for LF [86]. The unfolding, zinc release, and zinc accessibility transition midpoints for each enzyme are summarized in Table 3.9. It is clear from the data that the structural transitions differ for each zinc enzyme. Indeed, LF was previously found to unfold at relatively low concentrations of the denaturant GdnHCl (\(C_{\text{mid}} = 0.5\) M) [86]. In addition, LF was capable of retaining its catalytic zinc ion even when unfolded, as evidenced by the midpoint of 3.1 M for the metal release transition [86]. Such discrepancy in the midpoint concentrations was also observed for TL, where 50% of Zn\(^{2+}\) was released at 4.9 M GdnHCl, a concentration clearly beyond that required for TL unfolding (\(C_{\text{mid}} = 1.8\) M). Furthermore, a virtually identical midpoint concentration was observed for the analysis of the accessibility of TL’s catalytic zinc ion towards
chelation by PAR. This observation is in contrast to that reported for LF. Zinc accessibility studies for LF revealed a transition (with $C_{\text{mid}}$ of 1.0 M) in between those documented for the unfolding and metal release [86]. This Zn$^{2+}$ accessibility transition likely arises from a structural change in LF, where the enzyme converts from a PAR-inaccessible (closed) state to a PAR-accessible (open) form, thus allowing the chelator to gain access to the Zn$^{2+}$ ion in the active site (prior to the metal’s spontaneous release, which occurs at much higher GdnHCl concentrations). In the case of TL, it therefore appears that a similar transition does not exist since the conversion of the PAR-inaccessible state leads directly to a state of the enzyme that allows for the spontaneous release of the metal ion. As for CPA, the unfolding of the enzyme followed a three-state mechanism ($N \rightarrow I \rightarrow U$), in contrast to the two-state unfolding mechanism observed for LF and TL. Similar to LF (but in contrast to TL), the metal accessibility and release midpoints for CPA differ. Interestingly, CPA’s $N \rightarrow I$ transition appears to coincide with the zinc accessibility transition, whereas the transition from the I to the U state seems to correlate with metal release. This observation supports the notion of enzymes (such as LF and CPA) converting from a PAR-inaccessible (closed) form to a PAR-accessible (open) state with increasing concentrations of denaturant. Based on these results, it is plausible that PAR can serve as a useful zinc accessibility probe (ZAP) to study the (un)folding pathways of other metalloproteins.
Table 3.9: Summary of $C_{\text{mid}}$ values from the unfolding, zinc accessibility, and zinc release studies for LF, TL, and CPA.

<table>
<thead>
<tr>
<th>Transition</th>
<th>$C_{\text{mid}}$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LF$^a$</td>
<td>TL</td>
</tr>
<tr>
<td>Unfolding</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc Accessibility</td>
<td>1.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Zinc Release</td>
<td>3.1</td>
<td>4.9</td>
</tr>
</tbody>
</table>

$^a$ Values were taken from [86].

$^b$ Values were obtained from the GdnHCl-induced CPA unfolding titration in the presence of ANS.
3.6 Conclusions and future studies

Previous studies have shown that LF retains its catalytic Zn$^{2+}$ ion even when unfolded (in GdnHCl), and that the enzyme’s active site zinc ion becomes accessible to chelation by PAR prior to the spontaneous release of the metal ion [86]. This chapter was aimed at determining whether similar structural transitions occur with other zinc proteases, such as TL and CPA, and whether PAR has the potential to serve as a zinc accessibility probe (ZAP) to study structural transitions in these enzymes.

The results described in this section show that the protein unfolding, zinc accessibility and release transitions all differ for LF, TL, and CPA. Similar to LF, TL unfolded in the presence of relatively low GdnHCl concentrations. However, the metal release and accessibility transitions were identical. In addition, an undocumented intermediate state was observed in the unfolding pathway of CPA. Interestingly, the zinc accessibility midpoint concentration coincides well with the C$_{mid}$ value for the N → I unfolding transition, whereas the zinc release transition corresponds to the I → U unfolding transition. It is therefore clear that the structural transitions (i.e., protein unfolding and metal dissociation) differ for each zinc protease studied in this chapter. These results demonstrate the potential applicability of PAR as a ZAP for the study of protein (mis)folding. As such, ZAPs might be of value in future investigations to gain further insight into the mechanisms underlying the (metal-mediated) misfolding of proteins involved in a variety of neurodegenerative diseases, including Alzheimer’s disease, prion diseases, and amyotrophic lateral sclerosis [155]. In fact, mutations in Cu/Zn-superoxide dismutase (SOD1) cause approximately 20% of cases of the familial form of ALS, a lethal disease characterized by the relentless death of motor neurons in the brain and spinal cord [156]. The mechanism by which
mutant SOD1 directly causes ALS is unknown. However, it has been found that there is a possible role for SOD1 misfolding in ALS [132, 156]. Therefore, PAR could be used as a structural probe to investigate the mechanistic aspects of SOD1 (mis)folding.

Future studies could be pursued to further investigate the structural transitions in CPA, using circular dichroism (CD) spectroscopy. Although the fluorescent probe ANS was quite useful in the characterization of CPA’s MG intermediate state, the structural information gathered from such studies is rather limited. Therefore, data from CD measurements would complement the findings reported here, and would further probe the structural transitions occurring upon the GdnHCl-induced unfolding of CPA. In addition, the nature of the aggregates observed upon the renaturation of CPA (U → N and I → N) could be investigated using dynamic light scattering (DLS). DLS would provide information on the aggregate sizes, distribution widths, concentration, and lifetime [157]. Furthermore, experiments similar to those described in this section could also be performed with the non-ionic denaturant urea, or other chromophoric chelators such as Zincon [158] or derivatives of PAR. Lastly, the applicability of PAR to study structural transitions could be determined with other zinc proteins, including SOD1 and carbonic anhydrase (CA). SOD1 catalyzes the dismutation of the superoxide radical to molecular oxygen and hydrogen peroxide, with the zinc ion being thought to maintain the structural integrity of the active site [23]. On the other hand, CA, the first zinc metalloenzyme discovered, catalyzes the reversible hydration of carbon dioxide [11]. Both zinc enzymes described in this chapter (TL and CPA), as well as LF, are peptidases. Therefore, it would be valuable to perform similar experiments (i.e., GdnHCl-induced unfolding, metal release, and metal accessibility by PAR) with non-peptidases such as SOD1 and
CA. Such experiments would provide further insight into the usefulness of PAR as a ZAP to investigate structural transitions in zinc proteins.
4. Design of a New Fluorescent Substrate for TL and TL-like Proteases

4.1 Background

The industrially important zinc-dependent endopeptidase thermolysin (TL) is the prototype of the M4 family of metallopeptidases [58, 59]. The activity of TL and its relatives is most frequently assessed using the short furylacryloyl peptide N-furylacryloylglycyl-L-leucinamide (FAGLA). The hydrolysis of FAGLA is usually monitored spectrophotometrically at 345 nm [93] or at 322 nm [92]. However, a very small change in the extinction coefficients ($\Delta\varepsilon_{345\text{ nm}} = 317 \text{ M}^{-1} \text{ cm}^{-1}, \Delta\varepsilon_{322\text{ nm}} = 2300 \text{ M}^{-1} \text{ cm}^{-1}$) poses a limit on the sensitivity of the assay [92, 93]. Moreover, the Michaelis constant ($K_M$), a value representing the substrate concentration at which the reaction rate is half of its maximal value, at neutral pH cannot be determined due to the low solubility of the peptide in buffered media [91]. Therefore, most assays with FAGLA have been performed under pseudo-first order conditions (i.e., at substrate concentrations much lower than $K_M$ and its solubility limit). Although these conditions do not allow for the determination of the individual parameters $k_{cat}$ (turnover number) and $K_M$, the catalytic efficiency ($k_{cat}/K_M$) can be assessed [91, 159, 160].

To overcome the limitations with FAGLA, many synthetic peptides coupled to fluorescence dyes have been developed over the past decade to monitor the activity of TL-like proteases via Förster resonance energy transfer (FRET). In the uncleaved peptide, the excitation energy is transferred from an excited fluorescent donor to a vicinal quenching acceptor [126]. Cleavage of the internally quenched fluorogenic peptide results in spatial separation of the fluorophore and the quencher, thus producing a large increase in fluorescence intensity [126].
Many donor-acceptor FRET pairs have been reported for a variety of peptide substrates, including 2-aminobenzoyl (Abz)/3-nitrotyrosine, Abz/2,4-dinitrophenyl (Dnp), (7-methoxycoumarin-4-yl)acetyl (Mca)/Dnp, 4-(4'-N,N-dimethylaminophenyl)azobenzoyl (Dabcyl)/fluorescein isothiocyanate (FITC), and 5-(dimethylamino)-naphthalenyl-1-sulfonyl (Dns)/tryptophan (see Figure 4.1) [161].

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="3-Nitrotyrosine" /></td>
<td><img src="image2" alt="Abz" /></td>
</tr>
<tr>
<td><img src="image3" alt="Dabcyl" /></td>
<td><img src="image4" alt="Dnp" /></td>
</tr>
<tr>
<td><img src="image5" alt="Dns" /></td>
<td><img src="image6" alt="EDANS" /></td>
</tr>
<tr>
<td><img src="image7" alt="Mca" /></td>
<td><img src="image8" alt="FITC" /></td>
</tr>
</tbody>
</table>

**Figure 4.1: Structures of common FRET systems.**
Besides the aforementioned donor/acceptor systems, the combination of the donor fluorophore \(N\)-(2-aminoethyl)aminonaphthalene-5-sulfonic acid (EDANS) (see Figure 4.1) and the acceptor chromophore Dabcyl, is one of the most frequently used FRET pairs due to the excellent overlap of the donor/acceptor wavelengths, and the large increase in donor emission that can be observed upon donor/acceptor separation [161-164]. Weimer and coworkers have previously synthesized the short FRET dipeptide Dabcyl-SF-EDANS to assess the activity of TL, revealing a moderately high \(K_M\) value of 104 \(\mu\)M, and a relatively low \(k_{cat}/K_M\) value of \(4.1 \times 10^3\) M\(^{-1}\) s\(^{-1}\) [94].

As previously mentioned, TL preferentially cleaves substrates bearing a hydrophobic/bulky amino acid residue (e.g., Phe, Ile, Leu, Val) at the P1’ position, and a Phe residue at the P1 position [74]. In addition, TL has a preference for a Lys at the P3’ position, likely due to the favourable electrostatic interactions with the carboxylate side chain of Asp213 located in the enzyme’s substrate-binding pocket [95]. The substrate specificity of TL beyond the cleavage site (P3 to P3’) was further examined by Oda and coworkers using FRET libraries [95]. The authors found TL to prefer Leu at the P1’ position, Gly >> Arg > Asp at the P2’ position, and Lys at the P3’ position (as described above). Furthermore, the optimal residues at the P1 position were found to be Phe, Leu, Met, and Tyr, whereas Lys and Tyr were preferred at the P2 position, and Phe and Val at the P3 position. Overall, the authors revealed TL’s optimal recognition sequence (from P3 to P3’) to be Phe-Lys-Phe-Leu-Gly-Lys [95].
4.2 Objectives and Hypotheses

The objective of this study is to design a FRET-quenched peptide using the Dabcyl/EDANS FRET system described by Weimer and coworkers [94], and to extend the amino acid sequence by integrating TL’s specificity for the sequence Phe-Lys-Phe-Leu-Gly-Lys (P3 to P3’) in order to increase both the substrate affinity and catalytic efficiency. Hence, this work is aimed at generating a novel substrate for TL (Dabcyl-FKFLGKE-EDANS; see Figure 4.2) with which the enzyme’s activity and inhibition can be assessed in a more sensitive manner.

![Figure 4.2: Overall structure of the Dabcyl-FKFLGKE-EDANS peptide.](image)

The N-terminal Dabcyl quenching acceptor, the C-terminal EDANS fluorescence donor, and TL’s specificity for the sequence FKFLGK (P3 – P3’) are integrated in the peptide. The C-terminal Glu7 residue was required to label the peptide with the EDANS group.

Following initial assay optimization, the cleavage site within the substrate will be ascertained. The products of the hydrolysis reaction after exposure of the heptapeptide to the enzyme will be observed using electrospray ionization mass spectrometry (ESI-MS). The substrate affinity and catalytic efficiency will be determined by monitoring the initial rates of TL-mediated Dabcyl-FKFLGKE-EDANS hydrolysis as a function of substrate concentration using fluorescence
spectroscopy. The effects of temperature and pH on the rate of peptide cleavage will be assessed by monitoring the initial rates at a variety of temperatures and pH values. Finally, the propensity of proteases other than TL (including serine, cysteine, and zinc proteases) to cleave the substrate will be determined.

It is hypothesized that extending the length of an EDANS/Dabcyl-modified peptide substrate to integrate TL’s specificity for the sequence Phe-Lys-Phe-Leu-Gly-Lys (P3 to P3’) will increase both the substrate affinity and catalytic efficiency. It is further hypothesized that:

1. TL cleaves the new substrate at the intended position (i.e., between Phe3 and Leu4),
2. The activity of the enzyme is maximal between 60 and 70 °C,
3. TL will display maximal activity at a near-neutral pH (between 6 and 7)
4. Dispase, a TL-like protease possessing a substrate specificity similar to that of TL, will efficiently hydrolyze the peptide, and
5. Serine, cysteine, and other zinc proteases show negligible or significantly lower activities towards the substrate.
4.3 Materials and Methods

4.3.1 Chemicals and laboratory equipment

Acetonitrile, formic acid, and dimethyl sulfoxide (DMSO) were purchased from Caledon Laboratories Ltd. (Georgetown, ON). Ammonium acetate and 2-(N-morpholino)ethanesulfonic acid (MES) were obtained from BioShop (Burlington, ON). Lastly, ammonium hydroxide was purchased from Stanchem (Vancouver, BC). All other chemicals used in the work described in this chapter are listed in Table 3.1 (section 3.3.1). The laboratory equipment used in the studies outlined below is identical to that shown in Table 3.2 (section 3.3.1).

4.3.2 Stock solutions

All aqueous solutions were prepared using Milli-Q ultrapure water ($\geq 18.2$ MΩ cm resistivity). HEPES buffer (50 mM, pH 7.4) was prepared as outlined in section 3.3.2.

AMT is a three-component buffer consisting of acetic acid, MES, and Tris, and is frequently used in pH studies to maintain a constant ionic strength in the pH range of 4.0 to 9.0 [165]. A 200 mM AMT stock solution was prepared by dissolving 572 µL of acetic acid (0.1 M), 1.952 g of MES (0.1 M), and 2.423 g of Tris (0.2 M) in 100 mL water. An 8-fold dilution of the AMT stock solution (25 mM; ionic strength = 25 mM) resulted in acetic acid ($pK_a$ of 4.75), MES ($pK_a$ of 6.15), and Tris ($pK_a$ of 8.1) final concentrations of 12.5 mM, 12.5 mM, and 25 mM, respectively. AMT buffer was kept at room temperature.

Ammonium acetate buffer (10 mM, pH 7.0) was prepared by dissolving 0.385 g of ammonium acetate in 500 mL water. The pH was adjusted with 1 M ammonium hydroxide (NH$_4$OH), and the buffer was stored at room temperature.
4.3.3 Preparation of enzyme and substrate solutions

TL and CPA stock solutions were prepared as outlined in section 3.3.3. Papain (EC 3.4.22.2) from papaya latex as well as the bovine pancreatic enzymes chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4) were obtained from Millipore Sigma (St. Louis, MO), and were used without further purification. Dispase (neutral protease; EC 3.4.24.28) from *Paenibacillus polymyxa* was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Anthrax lethal factor (EC 3.4.24.83) was expressed in *Bacillus megaterium*, and purified according to published procedures [166].

Stock solutions of papain were obtained by dissolving 1 mg of the enzyme in 0.5 mL of water. The concentration of papain in these solutions was estimated spectrophotometrically at 280 nm using an extinction coefficient of 57 630 M\(^{-1}\) cm\(^{-1}\) [108]. Stock solutions of trypsin were obtained by dissolving 1 mg of the enzyme in cold HCl (1 mM) [167]. Stock solutions of chymotrypsin were prepared analogously except for the supplementation of the acidic medium with 2 mM CaCl\(_2\) [94, 168]. The concentrations of trypsin and chymotrypsin were determined at 280 nm using extinction coefficients of 37 670 M\(^{-1}\) cm\(^{-1}\) and 49 610 M\(^{-1}\) cm\(^{-1}\) [108], respectively. Stock solutions of dispase were prepared by dissolving 1 mg of the enzyme in 0.5 mL of HEPES buffer (50 mM, pH 7.4) containing 2 mM CaCl\(_2\) [94]. An extinction coefficient \(\varepsilon_{280\ \text{nm}}\) of 49 280 M\(^{-1}\) cm\(^{-1}\) (calculated based on the amino acid sequence of the protein [169]) was employed to determine the concentration of dispase in these solutions. All enzyme stock solutions were stored in -80 °C.

Dabcyl-FKFLGKE-EDANS (with the EDANS moiety attached to the peptide via the \(\gamma\)-carboxylate of Glu7) was custom-synthesized by BioMatik Corporation (Cambridge, ON). Stock
solutions of the peptide (2 mM) were prepared by dissolving approximately 2 mg of peptide in 0.5 mL DMSO. The stock solution was then further diluted to 0.5 mM in DMSO. The concentration of the peptide was ascertained spectrophotometrically (based on the absorption of the Dabcyl chromophore) at 450 nm using an extinction coefficient of 32 000 M\(^{-1}\) cm\(^{-1}\) [170]. Stock solutions were covered in aluminum foil, stored at -20 °C, and were found to be stable for at least one month.

4.3.4 Fluorescence spectroscopy

Fluorescence excitation (\(\lambda_{em} = 490\) nm) and emission (\(\lambda_{exc} = 336\) nm) spectra of fully hydrolyzed Dabcyl-FKFLGKE-EDANS (5 µM) in HEPES buffer (50 mM, pH 7.4) following exposure of the peptide to TL (5 nM), were recorded in a 10 × 4 mm quartz cell in 1 nm increments (integration time of 1 s) at room temperature using an OLIS RSM1000 spectrofluorometer (Bogart, GA) equipped with a 150 W xenon arc lamp and a photon counter. The excitation and emission bandpasses were set to 5 and 4 nm, respectively. All spectra were baseline-subtracted, and smoothed using the OLIS GlobalWorks software.

4.3.5 TL enzyme assays and determination of kinetic parameters

In a typical assay (total volume of 1 mL), TL (5 nM) in HEPES buffer (50 mM, pH 7.4) containing CaCl\(_2\) (10 mM) was supplemented with Dabcyl-FKFLGKE-EDANS (5 µM). Substrate hydrolysis was monitored at 490 nm (\(\lambda_{exc} = 336\) nm) for 60 s (1 s intervals) in a 10 × 4 mm quartz cell using the OLIS RSM1000 spectrofluorometer. Due to an inner filter effect observed at a substrate concentration of 15 µM, the initial velocities (with 15 µM substrate) were corrected by
a factor of 1.074. This value was determined based on the absorbance of the peptide at both the excitation and emission wavelengths as outlined in the literature [126, 127].

The linear portion of the progress curve (~30 s) was used to determine the initial velocities. The fluorescence intensity of the fully hydrolyzed peptide (5 µM) served as a reference to obtain the rates (expressed in nM of substrate hydrolyzed per second), as shown in eq (9),

$$v_c \left( \frac{nM}{s} \right) = \frac{5000 \ nM \times v_F \left( \frac{cps}{s} \right)}{(FI_p - FI_s)}$$

(9)

where $v_c$ and $v_F$ are concentration- and fluorescence intensity-based initial velocities, respectively, and $FI_p$ and $FI_s$ are the fluorescence intensities (in cps) of 5 µM fully hydrolyzed and unhydrolyzed substrate, respectively.

Kinetic parameters ($K_M$ and $k_{cat}$) were determined by non-linear least squares fitting of the initial rates to the Michaelis-Menten equation (see eq (10)) using GraFit 4.0 (Erithacus Software Ltd., Staines, UK),

$$v_o = \frac{v_{max} \ [S]}{[S] + K_M}$$

(10)

where $v_o$ is the initial rate, $v_{max}$ is the maximum velocity, $[S]$ is the substrate concentration, and $K_M$ is the Michaelis constant. A Hanes-Woolf plot, which employs a linearized form of the
Michaelis-Menten equation was also obtained to graphically represent the data (see eq (11) \([171, 172]\)).

\[
\frac{[S]}{v_o} = \frac{[S]}{v_{max}} + \frac{K_M}{v_{max}}
\]  

(11)

**4.3.6 pH and temperature dependence**

The effect of pH on TL-mediated hydrolysis of Dabcyl-FKFLGKE-EDANS was assessed in a manner analogous to that outlined above, except for the replacement of HEPES with 25 mM AMT buffer, which allows for the maintenance of a constant ionic strength of 25 mM in the studied pH range (pH 4.0 to 9.0) \([165]\). Measurements were performed at low (50 \(\mu\)M) and high (10 mM) CaCl\(_2\) concentrations.

Assays to assess the temperature dependence (ranging from 25 to 80 °C) on the rate of peptide hydrolysis at pH 7.4 were performed following the addition of TL (5 nM) to 5 \(\mu\)M substrate in 50 mM HEPES buffer containing 10 mM CaCl\(_2\). The final pH value of 7.4 at the desired temperature was confirmed for all buffer solutions by taking into account the temperature dependence of the \(pK_a\) value of HEPES \((d(pK_a)/dT = -0.014) [173]\). The substrate-buffer mixture was pre-heated to the desired temperature in a 10 x 10 mm mirrored quartz cell prior to the supplementation with the enzyme. Upon addition of TL, the progress of substrate hydrolysis was monitored at 490 nm \((\lambda_{exc} = 336 \text{ nm})\) for 60 s (1 s intervals). A Pt100 probe connected to a Julabo CF31 water bath (Julabo, Seelbach, Germany) was used to confirm the temperature of the
solution, both before supplementation with the enzyme and after substrate hydrolysis. It was found that the temperature was always within ±0.5 °C of the desired temperature.

4.3.7 Estimation of the detection limit

The rate of hydrolysis of 5 µM Dabcyl-FKFLGKE-EDANS in the presence of 10 pM TL was measured at 490 nm (λ_{exc} = 336 nm) in HEPES buffer (50 mM, pH 7.4; + 10 mM CaCl₂), which was supplemented with 0.1 mg/mL bovine serum albumin to minimize adsorption of the enzyme to the quartz cell [174]. In addition, the spontaneous (uncatalyzed) hydrolysis of 5 µM Dabcyl-FKFLGKE-EDANS was monitored in HEPES buffer (50 mM, pH 7.4). Both measurements were performed three independent times to obtain the average rates and the standard errors of the mean (SEM; n=3). A statistical difference (95% confidence interval) between the rates for the spontaneous and TL-mediated hydrolysis rates was ascertained using the overlap rule for standard error bars (i.e., a statistically significant difference is observed when the errors bars representing 2 × SEM do not touch or overlap) [175].

4.3.8 Substrate hydrolysis by various proteases

The rate of substrate hydrolysis was measured with proteases other than TL, using the following assay conditions: dispase (5 nM) in HEPES buffer (50 mM, pH 7.4; + 2 mM CaCl₂) [94]; LF (1 µM) in HEPES buffer (50 mM, pH 7.4) [32]; CPA (1 µM) in Tris buffer (25 mM, pH 7.5; + 0.5 M NaCl) [109, 110]; chymotrypsin (1 µM) in HEPES buffer (50 mM, pH 7.4; + 2 mM CaCl₂) [168, 176]; trypsin (1 µM) in HEPES buffer (50 mM, pH 8.0; + 10 mM CaCl₂) [177]; and papain (0.1 µM) in citrate buffer (40 mM, pH 6.0; + 0.1 M L-cysteine) [178, 179]. Upon addition of the
enzyme to the substrate, the increase in fluorescence intensity at 490 nm ($\lambda_{ex} = 336$ nm) was monitored for 60 s. In the case of dispase, $K_M$ and $k_{cat}$ values were determined in a manner identical to that outlined for TL.

4.3.9 Mass spectrometry

A Xevo G2-XS Q-TOF mass spectrometer (Waters Corp., Milford, MA) was used to obtain the electrospray-ionization (ESI) mass spectra of Dabcyl-FKFLGKE-EDANS and its hydrolysis products, in a positive ion mode. The following parameters were set on the mass spectrometer: capillary voltage of 3 kV, cone voltage of 30 V, source temperature of 150 °C, desolvation gas temperature of 500 °C, desolvation gas flow rate of 1000 L/hr, and cone gas flow rate of 50 L/hr. The sample infusion rate was set to 10 µL/min and data was collected for 1 min. In addition, sodium iodide was used for calibration in the m/z range of 100-7000. Prior to measurement, a stock solution (2 mM) of Dabcyl-FKFLGKE-EDANS was prepared by dissolving 6 mg of the peptide in 2 mL of fresh ammonium acetate buffer (pH 7.0). The substrate solution was subsequently diluted to 0.5 mM with ammonium acetate buffer, and supplemented with TL, dispase (final concentration of 0.6 µM for both enzymes), or trypsin (6 µM). Following a 1 h incubation period, an equal volume of acetonitrile containing 0.2% (v/v) formic acid was added to the enzyme-substrate mixtures before samples were introduced into the mass spectrometer. Unhydrolyzed substrate was measured analogously, and served as a control.
4.4 Results

This chapter describes a new FRET-quenched heptapeptide for assaying TL and TL-like proteases. The current study sought to extend the length of an EDANS/Dabcyl-modified dipeptide substrate [94], and to integrate TL’s specificity for the sequence Phe-Lys-Phe-Leu-Gly-Lys (P3 to P3’) to increase both the substrate affinity and the catalytic efficiency [95]. The experiments described below were aimed at investigating the substrate cleavage site, the kinetic parameters $K_M$ and $k_{cat}$, the optimal pH and temperature, and the propensity of other proteases (zinc, serine, and cysteine proteases) to hydrolyze the substrate.

4.4.1 Dabcyl-FKFLGKE-EDANS spectra

Previous reports regarding the short FRET dipeptide Dabcyl-SF-EDANS [94] and TL’s preference for the sequence Phe-Lys-Phe-Leu-Gly-Lys (P3 to P3’) [95] provided inspiration for the generation of the peptide Dabcyl-FKFLGKE-EDANS to serve as a substrate for TL and TL-like proteases. The EDANS/Dabcyl pair is one of the most frequently used FRET systems to assess the activity of various proteases by fluorescence spectroscopy, in view of the excellent overlap of donor emission and acceptor absorption spectra, and the large increase in fluorescence intensity observed upon donor/acceptor separation (see Figure 4.3B) [161-164]. The absorption spectrum (showing a $\lambda_{max}$ at 450 nm in DMSO, $\varepsilon_{450\text{ nm}} = 32\,000\,\text{M}^{-1}\text{cm}^{-1}$) was found to overlap nicely with the fluorescence emission spectrum of the fully hydrolyzed peptide in HEPES buffer (50 mM, pH 7.4) ($\lambda_{max} = 490\,\text{nm}$). The $\lambda_{max}$ of the fluorescence excitation spectrum ($\lambda_{em} = 490\,\text{nm}$) of the fully hydrolyzed substrate was determined to be 336 nm. Therefore, as shown in Figure 4.3A, in its heptapeptidic form, the peptide is internally quenched due to the spatial proximity of the two
labels, and an increase in emission intensity at 490 nm ($\lambda_{\text{exc}} = 336$ nm) is observed upon hydrolytic cleavage of the peptide.

Figure 4.3: Schematic representation of the fluorescence enhancement by Dabcyl-FKFLGKE-EDANS hydrolysis. (A) The Dabcyl group internally quenches the emission of EDANS when the two labels are in close proximity. Once separated, excitation of the EDANS-labeled peptide moiety leads to an enhancement in emission at 490 nm ($\lambda_{\text{exc}} = 336$ nm). (B) Fluorescence excitation (blue; $\lambda_{\text{em}} = 490$ nm) and emission (green; $\lambda_{\text{exc}} = 336$ nm) spectra of fully hydrolyzed Dabcyl-FKFLGKE-EDANS (5 μM). The absorption spectrum of Dabcyl-FKFLGKE-EDANS recorded in DMSO is shown as a red dashed line.

4.4.2 Optimal assay conditions

Assay conditions using 5 μM Dabcyl-FKFLGKE-EDANS and 5 nM TL in Ca$^{2+}$-supplemented HEPES buffer (50 mM, pH 7.4) were found to be most suitable for monitoring TL activity. Under these conditions, a 30-fold fluorescence enhancement was achieved when exposing the enzyme to substrate for 5 min (see Figure 4.4). In addition, the linearity of the rate of substrate hydrolysis within the first 30 s allowed for the rapid assessment of initial velocities (see Figure 4.4).
Furthermore, the rate of the spontaneous uncatalyzed hydrolysis of Dabcyl-FKFLGKE-EDANS was found to be very small $(20 \pm 5 \text{ pM/s})$. In contrast, in the presence of TL (5 nM), a rate of 40 nM/s was obtained. Hence, the enzyme was found to accelerate the cleavage of the peptide by a factor of $2 \times 10^3$. Such rate enhancement could be exploited to detect significant (at a 95% confidence level) substrate turnover at TL concentrations as low as 10 pM in the assay, where the observed rate was $170 \pm 35 \text{ pM/s}$.

Figure 4.4: Reaction progress curve for TL-mediated hydrolysis of Dabcyl-FKFLGKE-EDANS. The spontaneous (uncatalyzed) hydrolysis of substrate is shown in red. The progress of substrate (5 µM) cleavage by TL (5 nM) in HEPES buffer (50 mM, pH 7.4; + 10 mM CaCl$_2$) is depicted in blue. The initial rate (extrapolated, and shown as a black line) was estimated from the linear portion of the curve (initial 30 s).
4.4.3 Determination of the cleavage site by ESI-MS

The products of the TL-catalyzed hydrolysis of Dabcyl-FKFLGKE-EDANS were determined by ESI-MS, in order to ascertain TL’s preferential cleavage site (i.e., between Phe3 and Leu4). As shown in Figure 4.5, peaks at m/z 1367.6, 684.3, and 456.6 were observed in the mass spectrum of the unhydrolyzed substrate, and were consistent with the theoretical m/z values of the intact peptide (for the [M+H]+, [M+H]2+ and [M+H]3+ species, respectively).

Figure 4.5: Mass spectrum of Dabcyl-FKFLGKE-EDANS. The substrate solution (2 mM) was diluted to 0.5 mM with ammonium acetate buffer (10 mM, pH 7) and introduced into the mass spectrometer.
The supplementation of TL to Dabcyl-FKFLGKE-EDANS resulted in the disappearance of the peaks associated with the unhydrolyzed peptide, and in the emergence of peaks at m/z 692.3 and 346.7 (indicative of the [M+H]\(^+\) and [M+2H]\(^{2+}\) charge states of the Dabcyl-FKF fragment), and a peak at m/z 694.3 (corresponding to the [M+H]\(^+\) charge state of the LGKE-EDANS moiety) (see Table 4.1 and Figure 4.6), thus confirming the expected cleavage site (between Phe3 and Leu4). Interestingly, a minor portion (~10%) of fragments corresponding to the Dabcyl-FK and FLGKE-EDANS fragments (m/z 545.3 and 841.4, respectively) were also observed in the mass spectrum (see Figure 4.6 and Table 4.1), thus suggesting that TL is capable of cleaving the peptide between Lys2 and Phe3 as well. This comes as no surprise considering TL has a preference for hydrophobic residues (i.e., Phe) at the P1’ position [74].

Table 4.1: Analysis of the Dabcyl-FKFLGKE-EDANS hydrolysis products by ESI-MS.

<table>
<thead>
<tr>
<th>Substrate/Fragment Sequence</th>
<th>Charge (z)</th>
<th>Experimental m/z TL</th>
<th>Theoretical m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dabcyl-FKF</td>
<td>+1</td>
<td>692.33</td>
<td>692.83</td>
</tr>
<tr>
<td></td>
<td>+2</td>
<td>346.68</td>
<td>346.92</td>
</tr>
<tr>
<td>LGKE-EDANS</td>
<td>+1</td>
<td>694.31</td>
<td>694.82</td>
</tr>
<tr>
<td>Dabcyl-Fk(^b)</td>
<td>+1</td>
<td>545.27</td>
<td>545.66</td>
</tr>
<tr>
<td>FLGKE-EDANS(^b)</td>
<td>+1</td>
<td>841.35</td>
<td>841.99</td>
</tr>
</tbody>
</table>

\(^a\) The results obtained with dispase will be discussed in section 4.4.7.
\(^b\) A minor amount (~10%) of cleavage products arising from the hydrolysis of the peptide bond between Lys2 and Phe3 was observed.
Figure 4.6: Mass spectrum of Dabcyl-FKFLGKE-EDANS after TL-catalyzed hydrolysis. The substrate solution (2 mM) was diluted to 0.5 mM with ammonium acetate buffer (10 mM, pH 7), and supplemented with TL (final concentration of 0.6 µM). The enzyme-substrate mixture was introduced into the mass spectrometer following 1 h of incubation.

4.4.4 Kinetic study of Dabcyl-FKFLGKE-EDANS hydrolysis by TL

At a substrate concentration of 15 µM (the highest concentration of the peptide used in the assessment of $K_M$ and $k_{cat}$), the onset of an inner filter effect was observed (see Figure 4.7). Therefore, the initial velocities recorded with 15 µM substrate were corrected by a factor of
1.074, which was determined based on the absorbance of the peptide at both the excitation and emission wavelengths as outlined in the literature [126, 127].

Figure 4.7: Dependence of fluorescence intensity on the concentration of hydrolyzed Dabcyl-FKFLGKE-EDANS. The emission intensity was monitored at 490 nm ($\lambda_{exc} = 336$ nm) following full hydrolysis of Dabcyl-FKFLGKE-EDANS (at the indicated concentrations) by TL (5 nM) in HEPES buffer (50 mM, pH 7.4). Deviations from the straight line at concentrations ≥15 µM are indicative of an inner filter effect.

The initial rates of TL-mediated Dabcyl-FKFLGKE-EDANS hydrolysis as a function of the substrate concentration were assessed, and were found to obey typical Michaelis-Menten behaviour. As shown in Figure 4.8, a decrease in the CaCl$_2$ concentration from 10 mM to 50 µM in the assay medium was not found to significantly alter the kinetic parameters of substrate hydrolysis. The $K_m$ values determined for the hydrolysis of Dabcyl-FKFLGKE-EDANS by TL with
10 mM and 50 μM Ca\(^{2+}\) were 3.95 (± 1.02) μM and 4.35 (± 0.39) μM, respectively. These low \(K_M\) values clearly suggest that TL has a much stronger affinity for Dabcyl-FKFLGKE-EDANS compared to the dipeptide Dabcyl-SF-EDANS (\(K_M = 104\) μM [94]). The \(k_{cat}\) values for the cleavage of the heptapeptide substrate in the presence of 10 mM and 50 μM Ca\(^{2+}\) were found to be 14.2 (± 1.1) s\(^{-1}\) and 15.9 (± 0.3) s\(^{-1}\), respectively. Thus, the \(k_{cat}/K_M\) values were determined to be 3.6 (± 0.9) × 10\(^6\) M\(^{-1}\)s\(^{-1}\) (10 mM Ca\(^{2+}\)) and 3.7 (± 0.3) × 10\(^6\) M\(^{-1}\)s\(^{-1}\) (50 μM Ca\(^{2+}\)), clearly exceeding that reported for Dabcyl-SF-EDANS by three orders of magnitude [94]. The observation that the kinetic parameters determined at low Ca\(^{2+}\) concentrations were very similar to those obtained with 10 mM Ca\(^{2+}\) indicates that for the duration of the assay, TL is stable even at low concentrations (50 μM) of the alkaline earth metal ion.

![Figure 4.8: Michaelis-Menten plots of TL-mediated Dabcyl-FKFLGKE-EDANS hydrolysis at high (10 mM) and low (50 μM) CaCl\(_2\) concentrations.](image)

TL (5 nM) in HEPES buffer (50 mM, pH 7.4) with (A) 10 mM CaCl\(_2\), or (B) 50 μM CaCl\(_2\) was supplemented with the substrate (S) at the indicated concentrations prior to recording the increase in emission intensity at 490 nm (\(\lambda_{exc} = 336\) nm). The line denotes the best fit of the data to the Michaelis-Menten equation, with the Hanes-Woolf replot depicted in the inset. Values shown represent the mean (±1 s.d.) of three independent experiments.
4.4.5 pH dependence

The influence of pH on the TL-mediated hydrolysis of the peptide was investigated in AMT buffer at low and high concentrations of Ca\(^{2+}\). As shown in Figure 4.9, at high Ca\(^{2+}\) concentrations (10 mM), a typical bell-shaped curve with maximal activities near pH 6.5 was observed. This result is in agreement with those obtained for the TL-catalyzed hydrolysis of a variety of other substrates, where pH optima between 6.0 and 7.0 have been reported [159, 180-182].

![Figure 4.9: pH dependence of Dabcyl-FKFLGKE-EDANS hydrolysis by TL](image-url)

**Figure 4.9: pH dependence of Dabcyl-FKFLGKE-EDANS hydrolysis by TL.** TL (5 nM) in AMT buffer (25 mM, at the desired pH) was supplemented with 5 \(\mu\)M substrate prior to recording the increase in emission at 490 nm (\(\lambda_{exc} = 336\) nm). Measurements were performed at CaCl\(_2\) concentrations of 50 \(\mu\)M (circles) and 10 mM (squares). Values shown represent the mean (±1 s.d.) of three independent experiments.
As shown in Figure 4.9, the pH titration profile remained unaltered in the (near-)neutral and alkaline regions at low Ca\(^{2+}\) concentrations (50 µM). However, under acidic conditions (pH < 5.5), proteolytic activities were clearly diminished with respect to those observed at high Ca\(^{2+}\) concentrations. Considering the reduced affinity of TL for Ca\(^{2+}\) at low pH [183], this observation might indicate that some of the Ca\(^{2+}\)-binding sites were depopulated, leading to a loss of structural integrity of the enzyme.

### 4.4.6 Temperature dependence

As shown in Figure 4.10, the influence of temperature on the hydrolysis of Dabcyl-FKFLGKE-EDANS by TL in the presence of 10 mM Ca\(^{2+}\) was explored. The maximal activity was observed at 65 °C (accompanied by a 3-fold increase in activity from 25 °C), a value similar to that documented in the literature [99-101]. Furthermore, a sharp decline of activity was noted at T ≥ 70 °C (see Figure 4.10). Interestingly, at low concentrations of the alkaline earth metal ion (50 µM) and at a temperature of 80 °C, the enzyme was found to be virtually inactive (data not shown). Therefore, high Ca\(^{2+}\) concentrations (10 mM) in the assay medium are absolutely necessary to maintain TL in a catalytically active state at temperatures exceeding 70 °C. This finding is consistent with the well-documented role of Ca\(^{2+}\) in the structural stabilization of TL at high temperatures [70, 71].
Figure 4.10: Temperature dependence of TL-catalyzed Dabcyl-FKFLGKE-EDANS. The substrate (5 μM) in HEPES buffer (50 mM, pH 7.4; + 10 mM CaCl₂) was heated to the desired temperature prior to initiation of the assay by the addition of TL (5 nM final concentration). The increase in emission was monitored at 490 nm (λexc = 336 nm). Values shown represent the mean (±1 s.d.) of three independent experiments.

4.4.7 Hydrolysis of Dabcyl-FKFLGKE-EDANS by other proteases

The proteolytic cleavage of the heptapeptide by several proteases was studied in order to establish enzyme specificities towards this substrate. Dispase, a TL-like zinc metalloprotease, is a clinically relevant agent used for the gentle dissociation of tissues [169, 184, 185]. As shown in Table 4.2, the enzyme was capable of efficiently hydrolyzing the fluorogenic peptide. However, two other zinc proteases (carboxypeptidase A and anthrax lethal factor) did not hydrolyze Dabcyl-FKFLGKE-EDANS to a significant extent (see Table 4.2). The cysteine protease (papain) and the serine proteases (trypsin and chymotrypsin) were found to cleave the substrate, although
they displayed significantly lower activities than those recorded with TL or dispase. ESI-MS analysis of the partial hydrolysis of Dabcyl-FKFLGKE-EDANS by trypsin revealed the enzyme to cleave (to a small extent) the peptide bonds between Lys2 and Phe3, and between Lys6 and Glu7 (data not shown).

**Table 4.2: Hydrolysis of Dabcyl-FKFLGKE-EDANS by various proteases.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Buffer</th>
<th>Relative Activity (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermolysin</td>
<td>HEPES (50 mM, pH 7.4) + 10 mM CaCl$_2$</td>
<td>100 (± 13)</td>
</tr>
<tr>
<td>Dispase</td>
<td>HEPES (50 mM, pH 7.4) + 2 mM CaCl$_2$</td>
<td>75 (± 7)</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>Tris (25 mM, pH 7.5) + 0.5 M NaCl</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Anthrax lethal factor</td>
<td>HEPES (50 mM, pH 7.4)</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>HEPES (50 mM, pH 7.4) + 2 mM CaCl$_2$</td>
<td>1.7 (± 0.3)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>HEPES (50 mM, pH 8.0) + 10 mM CaCl$_2$</td>
<td>0.6 (± 0.1)</td>
</tr>
<tr>
<td>Papain</td>
<td>Citrate (40 mM, pH 6.0) + 100 mM L-Cysteine</td>
<td>3.7 (± 1.0)</td>
</tr>
</tbody>
</table>

$^a$ Activities shown were normalized to account for differences in the concentrations of the enzymes used in the assays (see section 4.3.8). Values represent the mean (±1 s.d.) of three independent experiments.

In view of dispase showing a high turnover rate, the kinetic parameters for peptide cleavage by this enzyme were determined, in a manner identical to that outlined for TL. As shown in Figure 4.11, the Michaelis-Menten plot reveals a low $K_M$ of 2.32 ± 0.11 µM. Similarly to TL, dispase shows a much stronger affinity for Dabcyl-FKFLGKE-EDANS in comparison to the dipeptide Dabcyl-SF-EDANS ($K_M = 91$ µM [94]). The $k_{cat}$ and $k_{cat}/K_M$ values were found to be $10.7$ (± 0.1) s$^{-1}$ and $4.6$ (± 0.2) $\times 10^6$ M$^{-1}$ s$^{-1}$, respectively, values similar to those noted for TL (see section 4.4.4). Furthermore, the turnover number and catalytic efficiency for the hydrolysis of the heptapeptide by dispase exceeds those reported for the Dabcyl-SF-EDANS substrate ($k_{cat} = 0.26$ s$^{-1}$ and $k_{cat}/K_M = 2.8 \times 10^3$ M$^{-1}$ s$^{-1}$ [94]). In addition, ESI-MS analysis of the product
fragments following dispase-mediated hydrolysis of Dabcyl-FKFLGKE-EDANS revealed the presence of both Dabcyl-FKF and LGKE-EDANS fragments (see Table 4.1). Thus, both dispase and TL cleave the substrate, as expected, between the Phe3 and Leu4 residues.

**Figure 4.11:** Michaelis-Menten plot of the dispase-mediated cleavage of Dabcyl-FKFLGKE-EDANS. Dispase (5 nM) in HEPES buffer (50 mM, pH 7.4; + 2 mM CaCl₂) was supplemented with the substrate (S) at the indicated concentrations prior to monitoring its hydrolysis at 490 nm ($\lambda_{exc} = 336$ nm). The line denotes the best fit of the data to the Michaelis-Menten equation, with the Hanes-Woolf replot depicted in the inset. Values shown represent the mean (±1 s.d.) of three independent experiments.
4.5 Discussion

TL is a zinc-dependent metalloendopeptidase, and is the prototype for the M4 family of zinc metalloproteases [58, 59]. As mentioned previously, the most commonly employed TL substrate is FAGLA [92, 93]. However, this furylacryloyl dipeptide has major drawbacks, such as its limited solubility, as well as a small change in absorptivity [92, 93], the latter feature contributing to a low assay sensitivity. The short FRET peptide Dabcyl-FK-EDANS has been used previously to assess the activity of TL. However, its $K_M$ value (104 µM) and catalytic efficiency ($4.1 \times 10^3$ M$^{-1}$ s$^{-1}$) are relatively poor [94]. Thus, the length of the EDANS/Dabcyl-modified peptide substrate was extended in this study to incorporate the enzyme’s specificity for the sequence Phe-Lys-Phe-Leu-Gly-Lys (P3 to P3’) [95], in order to increase both the substrate affinity and catalytic efficiency.

4.5.1 TL-catalyzed hydrolysis of various peptide substrates

Throughout the years, the activities of TL and TL-like proteases have been assessed using various methods, such as the analysis of cleavage fragments with ninhydrin [186, 187], isotopic labeling (usually with tritiated substrates) [188, 189], and mass spectrometry [190]. However, chromogenic and small fluorescent peptide substrates (although larger proteins such as fluorescein isothiocyanate-labelled casein have also been used [191]) appear to be the more popular choice for measuring TL’s catalytic function. The catalytic parameters for many chromogenic and fluorogenic substrates of TL have been determined, and are summarized in Table 4.3. Usually the catalytic efficiencies reported for the chromogenic substrates, including FAGLA, are low. The exception appears to be the arazoformyl dipeptide (ArN=NCO-LA-OH [180]),
which efficiently releases N\textsubscript{2}, CO\textsubscript{2} and anisole upon hydrolysis. Hence, the azo compound not only serves as an excellent substrate of TL (with the largest $k_{cat}$ value shown in Table 4.3), but also exhibits a relatively large $\Delta \varepsilon$ value of 19,500 M\textsuperscript{-1} cm\textsuperscript{-1} at 350 nm [180], a feature contributing to a high assay sensitivity.

The catalytic parameters for fluorogenic TL substrates are typically larger than those of chromogenic substrates, but there are considerable variations (by roughly two orders of magnitude; see Table 4.3). High affinities and catalytic efficiencies have been documented for both the bimane-modified tetrapeptide harbouring a Dabsyl quencher (Dabsyl-PGLA-NH(CH\textsubscript{2})\textsubscript{2}S-Bim [192]), and the nonapeptide labeled with a luminescent europium(III) complex and a black hole quencher (Eu-KKKGFSAKK-(BHQ2) [193]). The Dabcyl-FKFLGKE-EDANS peptide described in this chapter was found to display $K_M$ values similar to those reported for the bimane- and lanthanide-modified peptides. However, the heptapeptide possesses the highest $k_{cat}/K_M$ value amongst all TL substrates listed in Table 4.3. Such high catalytic efficiency could be exploited to detect concentrations as low as 10 pM TL in assays with Dabcyl-FKFLGKE-EDANS, a concentration 5-fold lower than that reported for the lanthanide-labeled peptide [193]).
Table 4.3: Kinetic parameters of TL-mediated hydrolysis of various peptide substrates.

<table>
<thead>
<tr>
<th>Substrate(^a)</th>
<th>(\lambda_{\text{exc}}) (nm)</th>
<th>(\lambda_{\text{em}}) (nm)</th>
<th>(K_M) ((\mu)M)</th>
<th>(k_{\text{cat}}) (s(^{-1}))</th>
<th>(k_{\text{cat}}/K_M) (M(^{-1}) s(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorogenic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dabcyl-FKFLGKE-EDANS</td>
<td>336</td>
<td>490</td>
<td>3.95 (± 1.02)</td>
<td>14.2 (± 1.1)</td>
<td>3.6 (± 0.9) × 10(^6)</td>
<td>This work</td>
</tr>
<tr>
<td>Abz-AGLA-Nba</td>
<td>340</td>
<td>415</td>
<td>140</td>
<td>283</td>
<td>2.02 × 10(^6)</td>
<td>[194]</td>
</tr>
<tr>
<td>Abz-RSVIK(Dnp)</td>
<td>320</td>
<td>420</td>
<td>33</td>
<td>7</td>
<td>2.10 × 10(^5)</td>
<td>[195]</td>
</tr>
<tr>
<td>Bim-SCH(_2)CO-FWL-OH</td>
<td>398</td>
<td>480</td>
<td>24</td>
<td>0.85</td>
<td>3.5 × 10(^4)</td>
<td>[196]</td>
</tr>
<tr>
<td>CF-PSVAGLAGGC(Cy5)-NH(_2)</td>
<td>450</td>
<td>520</td>
<td>17.6</td>
<td>7.82</td>
<td>4.44 × 10(^5)</td>
<td>[197]</td>
</tr>
<tr>
<td>Dabcyl-SF-EDANS</td>
<td>336</td>
<td>490</td>
<td>104</td>
<td>0.43</td>
<td>4.1 × 10(^3)</td>
<td>[94]</td>
</tr>
<tr>
<td>Dabsyl-PGLA-NH(CH(_2))(_2)S-Bim</td>
<td>400</td>
<td>480</td>
<td>2</td>
<td>2.1</td>
<td>1.1 × 10(^6)</td>
<td>[192]</td>
</tr>
<tr>
<td>DBDY-(Ile-INH)(_2)</td>
<td>320</td>
<td>410</td>
<td>1.91</td>
<td>0.046</td>
<td>2.43 × 10(^4)</td>
<td>[198]</td>
</tr>
<tr>
<td>Dns-AAFA</td>
<td>340</td>
<td>540</td>
<td>200</td>
<td>240</td>
<td>1.2 × 10(^6)</td>
<td>[199]</td>
</tr>
<tr>
<td>Eu-KKKGFSAKK-(BHQ2)</td>
<td>295</td>
<td>617</td>
<td>4.49</td>
<td>2.6</td>
<td>5.79 × 10(^5)</td>
<td>[193]</td>
</tr>
<tr>
<td>Glutaryl-AAF-4MeO2NA</td>
<td>390</td>
<td>475</td>
<td>550</td>
<td>101.4</td>
<td>1.84 × 10(^5)</td>
<td>[200]</td>
</tr>
<tr>
<td>Mca-PLGL(Dpa)AR-NH(_2)</td>
<td>328</td>
<td>393</td>
<td>&gt; 90</td>
<td>n.d.</td>
<td>7.50 × 10(^4)</td>
<td>[101, 201]</td>
</tr>
<tr>
<td><strong>Chromogenic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ArN=NCO-LA-OH</td>
<td>350</td>
<td>1400</td>
<td>1218</td>
<td>8.7 × 10(^5)</td>
<td>[180]</td>
<td></td>
</tr>
<tr>
<td>Fua-GL-NH(_2) (FAGLA)</td>
<td>322, 345</td>
<td>&gt; 3 000</td>
<td>&gt; 90</td>
<td>1.3 × 10(^4) – 3.0 ×10(^4)</td>
<td>[91, 92, 159, 160, 202]</td>
<td></td>
</tr>
<tr>
<td>Fua-FA-NH(_2)</td>
<td>345</td>
<td>83</td>
<td>5.7</td>
<td>6.9 × 10(^4)</td>
<td>[203]</td>
<td></td>
</tr>
<tr>
<td>ZAPM</td>
<td>224</td>
<td>350</td>
<td>2.73</td>
<td>7.8 × 10(^3)</td>
<td>[91]</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 4MeO2NA, 4-methoxy-2-naphthylamine; Abz, 2-aminobenzoyl; Ar, 4-methoxyphenyl; BHQ2, black hole quencher 2; Bim, 1,7-dioxo-2,5,6-trimethyl-1H,7H-pyrazolo[1,2-\(\alpha\)]-pyrazol-3-yl-methyl; CF, carboxyfluorescein; Dabsyl, dimethylaminoazobenzenesulfonfyl; DBDY, \(N,N'\)-diBoc-dityrosine; Dnp, 2,4-dinitrophenyl; Dns, 5-(dimethylamino)-naphthalen-1-sulfonfyl; Dpa, \(N^3\)-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Eu, europium(III)-bound modified terpyridine, Fua, \(N\)-furylacryloyl; INH, isoniazid; Mca, (7-methoxycoumarin-4-yl)acetyl; Nba, 4-nitrobenzylamide; ZAPM, \(N\)-carbobenzoxy-L-aspartyl-L-phenylalaninemethyl ester.
4.5.2 Protease specificity for Dabcyl-FKFLGKE-EDANS

Dispase, a TL-like protease, was found to be capable of efficiently hydrolyzing Dabcyl-FKFLGKE-EDANS. This finding is not surprising since the enzyme’s substrate specificity is similar to that of TL [94, 169, 204]. As previously outlined, TL is the prototype of the M4 family of metallopeptidases. In general, members of this family demonstrate a strong preference for (bulky) hydrophobic (e.g., TL, aureolysin, coccolysin, λ-toxin) and/or aromatic residues (e.g., pseudolysin, griselysin, vimelysin) at the P1’ position [60]. Since the P1’ site is the major determinant of the substrate specificity for M4 family members [205], it is likely that Dabcyl-FKFLGKE-EDANS can serve as a substrate for M4 proteases other than TL. In fact, the observation that dispase (a P1’ protease [94]) efficiently hydrolyzes the heptapeptide appears to be in line with this argument.

The zinc proteases anthrax lethal factor (LF) and carboxypeptidase A (CPA) did not hydrolyze Dabcyl-FKFLGKE-EDANS to a significant extent. Although LF and TL share the prototypical HExxH...E active site motif characteristic of TL-like enzymes [42], LF is a highly specific protease preferring mitogen-activated protein kinase kinases (isoforms 1–4, 6 and 7, containing a conserved positively-charged consensus sequence N-terminal to the cleavage site) as substrates [81, 82]. It is therefore not surprising that LF is incapable of hydrolyzing the FRET-quenched heptapeptide described in this study (see Table 4.2). As for CPA, the zinc exopeptidase preferentially removes C-terminal amino acid residues with aromatic or branched sidechains [44]. It is therefore possible that the enzyme is incapable of recognizing the bulky C-terminal EDANS-modified Glu residue of the peptide.
Two serine proteases (trypsin and chymotrypsin) and papain, a cysteine protease, were also investigated to assess their potential to hydrolyze Dabcyl-FKFLGKE-EDANS. In comparison to TL and dispase, all three enzymes were found to show a significantly reduced activity towards this fluorogenic substrate (see Table 4.2). Trypsin is known to prefer positively charged Lys and Arg residues at the P1 position [206]. It is therefore not surprising that ESI-MS analysis performed after partial hydrolysis of Dabcyl-FKFLGKE-EDANS by trypsin revealed a small amount of peptide fragments consistent with the cleavage of the peptide bonds between Lys2 and Phe3, and between Lys6 and Glu7. The residual activity observed with chymotrypsin likely arises from its strong preference for aromatic residues at the P1 position [206], with Phe1-Lys2 and Phe3-Leu4 being the probable sites for the scissile peptide bonds. On the other hand, papain exhibits a preference for bulky hydrophobic or aromatic residues at the P2 position [178], and a relatively broad P1’ specificity (with Phe, Arg and Lys preferred over other residues [207]). Since Dabcyl-FKFLGKE-EDANS contains sites that match these above-mentioned criteria (at F1K2|F3 and L4G5|K6), it is likely that papain hydrolyzes the heptapeptide (albeit slowly) at these positions. Taken together, it appears that TL-like proteases have a high specificity for Dabcyl-FKFLGKE-EDANS.
4.6 Conclusions and future studies

By redesigning a previously documented dipeptide (Dabcyl-SF-EDANS [94]) to incorporate TL’s specificity sequence (Phe-Lys-Phe-Leu-Gly-Lys [95]), a FRET-quenched peptide was obtained that displays one of the largest catalytic efficiencies reported for any TL substrate. In fact, TL hydrolyzes the fluorogenic heptapeptide Dabcyl-FKFLGKE-EDANS with a specificity constant three orders of magnitude higher than that previously described for Dabcyl-SF-EDANS. In addition, due to the assay’s high sensitivity, concentrations as low as 10 pM TL are detectable. Finally, the coupling of EDANS and Dabcyl to peptide backbones is commonly performed in the laboratory or by most companies specializing in custom-made peptides to generate FRET-quenched peptides. Therefore, the cost of Dabcyl-FKFLGKE-EDANS, and the assay as a whole, would be greatly reduced in comparison to assays utilizing bimane-, lanthanide-, and black hole quencher-modified peptides.

Dabcyl-FKFLGKE-EDANS can be utilized to conveniently and rapidly determine the catalytic activity of TL, dispase (a TL-like protease from Paenibacillus polymyxa exhibiting substrate specificities similar to that of TL [169, 204]), and presumably other M4 proteases. Future studies could be pursued on various peptidases of the M4 family, to investigate their ability to efficiently hydrolyze the FRET-quenched heptapeptide. A number of M4 proteases have been implicated in a variety of diseases, and are therefore putative drug targets [60]. For example, both λ-toxin from Clostridium perfringens and coccolysin from Enterococcus faecalis are proteases of the M4 family, and share a similar substrate specificity to that of TL (i.e., large hydrophobic residues at the P1’ position) [60]. Indeed, the TL-like λ-toxin degrades numerous host proteins and activates the precursors of potent clostridial toxins [208]. This enzyme is
capable of degrading proteins contributing to innate or adaptive immunity against infections, such as immunoglobulin G, complement C3 component, fibrinogen, fibronectin and α2-macroglobulin [208]. On the other hand, coccolysin is involved in several infections such as urinary tract infections, secondary bacteremia, and food poisoning [60]. In addition, coccolysin has been found to inactivate human endothelin-1 [209], and hydrolyze casein, gelatin, and hemoglobin [60]. Given that TL and the aforementioned enzymes share a similar substrate specificity, Dabcyl-FKFLGKE-EDANS might therefore be useful to assess the catalytic activity and mechanism of these enzymes, and to assist in the development of novel therapeutics.
5. References


