Assessment of dimer-monomer equilibrium of a cold-adapted alkaline phosphatase by urea gel electrophoresis and ion exchange chromatography

Hildur Lúðvíksdóttir

Faculty of Biochemistry
University of Iceland
2017
Assessment of dimer-monomer equilibrium of a cold-adapted alkaline phosphatase by urea gel electrophoresis and ion exchange chromatography

Hildur Lúðvíksdóttir

15 ECTS thesis submitted in partial fulfilment of a Baccalaureus Scientiarum degree in Biochemistry

Advisor
Bjarni Ásgeirsson

Co-advisor
Magnús Már Kristjánsson

Faculty of Biochemistry
School of Engineering and Natural Sciences
University of Iceland
Reykjavík, May 2017
Assessment of dimer-monomer equilibrium of a cold-adapted alkaline phosphatase by urea gel electrophoresis and ion exchange chromatography

15 ECTS thesis submitted in partial fulfilment of a B.Sc. degree in Biochemistry

Copyright © 2017 Hildur Lúðvíksdóttir
All rights reserved

Faculty of Biochemistry
School of Engineering and Natural Sciences
University of Iceland
Dunhagi 3
107, Reykjavík
Iceland

Telephone: 525 4000

Bibliographic information:
Hildur Lúðvíksdóttir, 2017, Assessment of dimer-monomer equilibrium of a cold-adapted alkaline phosphatase by urea gel electrophoresis and ion exchange chromatography, B.Sc. thesis, Faculty of Biochemistry, University of Iceland, pp 80.

Printing: Háskólaprent, Fálkagata 2, 107 Reykjavík
Reykjavík, Iceland, May 2017
Abstract

A certain focus has been placed on enzymes working under extreme conditions in order to understand how they manage to function compared to enzymes working at milder, more viable, conditions. In this study, alkaline phosphatase from the cold-adapted marine bacterium *Vibrio splendidus* (VAP) was examined. The enzyme is a homodimer having two identical subunits. The interaction between the subunits is particularly interesting for that they appear to "talk" and take turns in binding the substrate, i.e. the enzyme is considered to be a half site reactivity enzyme.

In this project, the installation of a transverse urea gradient electrophoresis method was introduced and used to examine the kinetics associated with the monomer-dimer exchange of the enzyme at both low and high urea concentration, i.e. in both precursor conditions for unfolding as well as in unfolding conditions. Two variants of the enzyme were used, namely *StrepTag*-tagged VAP and 2x FLAG-tagged VAP due to an extra six negatively charged C-terminal residues on each monomer in the latter type. The hypothesis was that the strength of the subunit association is variable depending on the variant and that the dimer association also is dependent on how tightly the magnesium ion in the enzyme reaction site is bound. Various versions of urea gels were made and the condition for the electrophoreses was optimized throughout the experiments in attempt to achieve the most accurate analysis of the unfolding process of the enzyme as possible.

The second hypothesis examined was the existence of a monomer-dimer equilibrium. To find out if such an equilibrium was dynamic under normal experimental conditions, ion exchange chromatography was performed on a mixture of the two aforementioned VAP variants that can be separated using a Q-Sepharose ion exchange column. The mixing of the variants and the possible formation of a hybrid enzyme would confirm the hypothesis that a monomer-dimer equilibrium exists. Such a hybrid enzyme (i.e. a StrepTag-FLAG dimer) would be separable from the non-hybrid enzyme variants on a Q-Sepharose ion exchange column. The results were promising and do not rule out the possibility of an equilibrium between the monomeric and dimeric form. However, further experiments are needed to verify it.
Útdráttur

Áhugi hefur lengi beinst að ensímum sem starfa við jaðaraðstaður til að fá skilið hvernig starfsemi þeirra er hattað samanborið við önnur ensím sem starfa við mildari og lífvænlegri, aðstæður. Í þessari rannsókn er alkálískur fosfatazi úr kuldakeru sjávarörverunni Vibrio splendidus skoðaður (VAP), en hann er tvíliða sem samsett er úr tveimur eins undireiningum. Starfsemi undireininganna er einkar áhugaverð að því leyti að þær virðast "tala saman" og vinna á víxl (e. half of site reactivity).

Í þessu verkefni var fengist við uppsætningu á ákveðinni rafdráttaraðferð sem innihélt þverlægan þvagefnis-stigul í hlaupi. Með henni var skoðuð sú hraðafæði sem tengist einliðu-tvíliðu jafnvægi (e. monomer-dimer exchange) ensímins bæði við lága og háa urea styrki, þ.e. við undanfara aðstæður afmyndunar og við afmyndandi aðstæður. Unnið var með tvö afbrigði ensímins, annars vegar Flag-tag VAP og hins vegar 2x FLAG-tagged VAP. Tilgátan var sú að tengsl undireininganna séu misstækt eftir því hvaða afbrigði eigi í hlut og að tengslin séu einnig háð því hversu fast magnesiumjónin í hverfstöð ensímins binst. FLAG-afbrigði hefur sex neikvætt hlaðnar aminósýrur á C-enda hvorrar einliðu. Ýmsar útgáfur af urea hlaupum voru gerðar og rafdráttaraðstæðurnar voru þróaðar á meðan á rannsókninni stöð í þeim tilgangi að ná sem nákvæmastri mynd af afmyndunarferli ensímins.

Önnur tilgáta sem könnuð var, er sú að jafnvægi eigi sér stað á milli einliðanna og tvíliðanna. Til að kanna það var notast við jónaskiptaskiljun á afbrigðunum tveimur sem búið var að blanda saman í lausn og síðan aðgrein á Q-Sepharósa skilju. Blöndun þeirra og hugsanleg myndun hybrið afbrigðis (þ.e. Strep-FLAG VAP tvennd), sem aðgreinaleg væri á jónaskiptaskilju, myndi stafesta það að einliðu-tvíliðu jafnvægi væri til staðar. Niðurstöðurnar lofa góðu og útiloka ekki að jafnvægi eigi sér stað á milli einliða og tvíliða, en ljóst er að frekari rannsóknar er þörf til að staðfesta að svo sé.
Contents

List of Figures ............................................................................................................................... ix

List of Tables ................................................................................................................................. xi

No table of figures entries found. Abbreviations ................................................................. xii

Acknowledgements ..................................................................................................................... xiii

1 Introduction ................................................................................................................................. 15
  1.1 Alkaline phosphatase .............................................................................................................. 15
  1.1 Vibrio alkaline phosphatase ................................................................................................. 15
  1.3 Cold-adaptation .................................................................................................................... 17
  1.4 Historical beginnings of transverse urea gradient gel electrophoresis ......................... 19
  1.5 Capabilities of TUG-GE and critical parameters ............................................................... 20
    1.5.1 Capabilities, advantages and limitations ....................................................................... 20
    1.5.2 Critical parameters ........................................................................................................ 21
  1.6 Analysis of TUG-GE results ................................................................................................. 22
  1.7 The aim of the project .......................................................................................................... 25

2 Materials and methods .............................................................................................................. 27
  2.1 Materials ............................................................................................................................... 27
  2.2 Protein purification ............................................................................................................... 27
    2.2.1 Purification of Vibrio alkaline phosphatase ................................................................ 27
    2.2.2 SDS-PAGE protein electrophoresis ........................................................................... 28
  2.3 Determination of protein concentration ............................................................................. 28
  2.4 Activity measurements ......................................................................................................... 28
  2.5 Experimental Set-up for TUG-GE ...................................................................................... 29
    2.5.1 Building of the experimental setup for TUG-GE ......................................................... 29
    2.5.2 Setup of the gel casting apparatus ............................................................................. 30
  2.6 Preparation of the gel solutions ............................................................................................ 30
  2.7 Casting of the urea gradient gels .......................................................................................... 31
    2.7.1 Casting of the transverse urea gradient gels (TUG-gels) ............................................. 31
    2.7.2 Casting of the dual 0/8 M urea gels ........................................................................... 32
  2.8 Preparation and performance of urea gel electrophoresis ................................................. 32
  2.9 Separation of VAP variants on a HiTrap QFF column ...................................................... 34

3 Results ....................................................................................................................................... 35
  3.1 Purification of VAP-ST2 on Strep-Tag affinity column ...................................................... 35
  3.2 Building of the apparatus and determination of ideal condition for TUG-GE ............... 35
    3.2.1 Building of the apparatus ............................................................................................. 35
    3.2.2 Determination of the ideal conditions for the TUG-GE of VAP ................................ 36
  3.3 The first electrophoreses ...................................................................................................... 36
3.3.1 The first TUG-GE performed on native VAP ........................................ 36
3.3.2 The first TUG-GE performed on denatured VAP .................................. 37
3.4 Improvements of the apparatus .................................................................. 38
3.5 The first electrophoreses using the improved apparatus: controls .................. 38
  3.5.1 Electrophoresis of α-amylase and myoglobin ......................................... 38
  3.5.2 Electrophoresis of native VAP at RT .................................................. 40
3.6 TUG-GE performed at 4°C ........................................................................ 41
  3.6.1 Electrophoreses of native VAP .............................................................. 41
  3.6.2 Electrophoresis of urea denatured VAP ............................................... 43
  3.6.3 Electrophoresis of Strep-tagged and FLAG-tagged VAP on DTT containing TUG-gels ................................................................. 44
  3.6.4 Electrophoresis of VAP on dual 0/8 M urea gels ...................................... 47
3.7 Timing of Strep-tagged and 2x FLAG tagged VAP elution on a Q-Sepharose column ................................................................. 52
3.8 Activity staining testing and analysis of the effects riboflavin has on VAP activity ........................................................................................................ 57
3.9 Experiments on TUG-gels lacking riboflavin ................................................ 59

4 Discussions ..................................................................................................... 61

References .......................................................................................................... 65

Appendix ............................................................................................................. 67
List of Figures

Figure 1.1 The catalytic mechanism of alkaline phosphatase .................. 16
Figure 1.2 A ribbon representation of Vibrio alkaline phosphatase .......... 18
Figure 1.3 A schematic representation of the unfolding curve of a protein undergoing rapid interconversion ............................................. 22
Figure 1.4 Schematic diagrams of unfolding/refolding patterns expected for monomeric and oligomeric proteins ............................................. 23
Figure 3.1 Trial TUG-GE of VAP performed at RT in 0.5 M Tris-acetate, pH 8.0 electrophoresis buffer ...................................................... 37
Figure 3.2 TUG-GE of bacterial α-amylase from Bacillus subtilis performed at RT in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer .......................... 39
Figure 3.3 TUG-GE of myoglobin from equine heart. performed at RT in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer ................................. 39
Figure 3.4 TUG-GE of VAP performed at RT in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer .................................................. 40
Figure 3.5 TUG-GE of VAP performed at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer .................................................. 41
Figure 3.6 TUG-GE of VAP performed at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer .................................................. 42
Figure 3.7 TUG-GE of VAP performed at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer .................................................. 43
Figure 3.8 TUG-GE of denaturized VAP performed at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer .................................................. 44
Figure 3.9 TUG-GE of VAP-ST2 performed on a gel containing 10 mM DTT, at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer ....................... 45
Figure 3.10 TUG-GE of 2x FLAG tagged VAP performed on a gel containing 10 mM DTT, at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer ....................... 46
Figure 3.11 Electrophoresis of VAP-ST2 performed on a dual gel ............. 48
Figure 3.12 Electrophoresis of denatured VAP-ST2 sample performed on a dual gel ................................................................. 49
Figure 3.13 Electrophoresis of VAP-ST2 sample performed on a dual gel ........ 50
Figure 3.14 Electrophoresis of VAP-ST2 performed on a dual gel ........................................ 51
Figure 3.15 Electrophoresis of VAP-ST2 performed on a dual gel ........................................ 52
Figure 3.16 The separation of Strep-tag and 2x FLAG tagged VAP on a Q-Sepharose column ................................................................. 53
Figure 3.17 A representative chromatograph of Strep-tagged VAP sample loaded on a Q-Sepharose column ................................................................. 54
Figure 3.18 A representative chromatograph of 2x FLAG tagged VAP sample loaded on a Q-Sepharose column ................................................................. 54
Figure 3.19 The separation of Strep-tagged VAP, 2x FLAG tagged VAP and a mixture of the two variants on a Q-Sepharose column ................................................................. 55
Figure 3.20 SDS-PAGE electrophoresis of selected peaks from the Q-Sepharose column experiment performed on a mixture of VAP-ST2 and 2x FLAG tagged VAP variants ................................................................. 56
Figure 3.21 PAGE electrophoresis of the flow-through samples from the Q-Sepharose column experiment performed with a mixture of VAP-ST2 and 2x FLAG tagged VAP ................................................................. 57
Figure 3.22 Testing of the function of the activity staining solution ........................................ 58
List of Tables

Table 3.1 Protein purification table for the VAP-ST2 purified ................. 35
Table 3.2 The effect of riboflavin on VAP activity ......................... 58
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>VAP</td>
<td><em>Vibrio</em> Alkaline phosphatase</td>
</tr>
<tr>
<td>VAP-ST2</td>
<td><em>Strep</em>-tagged wild-type alkaline <em>Vibrio</em> alkaline phosphatase</td>
</tr>
<tr>
<td>2x FLAG</td>
<td>Double C-terminal FLAG-tagged wild-type <em>Vibrio</em> alkaline phosphatase</td>
</tr>
<tr>
<td>TUG-GE</td>
<td>Transverse urea gradient gel electrophoresis</td>
</tr>
<tr>
<td>TUG-gels</td>
<td>Transverse urea gradient gels</td>
</tr>
<tr>
<td>TMC</td>
<td>Tris-magnesium-chloride</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature (20-25°C)</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
Acknowledgements

I want to thank my instructor Prof. Bjarni Ásgeirsson for the opportunity to work on this project as well as his excellent guidance and insight during the course of the project. I also want to thank Jens Guðmundur Hjörleifsson for providing me with the VAP variants and for his helpful input and advice regarding the project. Finally, I want to thank Sigtryggur Bjarki Sigtryggsson, my family and friends for their unconditional support during the studies.
1 Introduction

1.1 Alkaline phosphatase

Alkaline phosphatases (APs) are enzymes that are widely distributed in nature and occur in a wide range of organisms ranging from bacteria to men. They function as extracellular enzymes catalyzing the hydrolysis or transesterification of phosphoryl esters under alkaline conditions. They display broad substrate selectivity and generally have a dimeric structure (Asgeirsson & Andresson, 2001; Millan, 2006). Each monomer has a α/β protein structure where the β-sheet can be found buried in the center of each monomer, while the α-helices are located on each side of the β-sheet (Kim & Wyckoff, 1991). In the active site of each monomer there is a nucleophilic serine residue along with three conserved metal ion binding sites, one for Mg$^{2+}$ and two for Zn$^{2+}$ ions. The metal ions are necessary and play an important role for both the enzyme’s stability and its catalytic activity (Asgeirsson & Andresson, 2001; Millan, 2006).

While the structural details of bacterial APs in various organisms varies a lot not only in sequence but the size of the polypeptide chains too, the core active site region is highly conserved (Helland et al., 2009). The amino acids that are particularly well conserved are those who interact with the metallic ions as well as the nucleophilic serine residue and an arginine (R129 in VAP) which binds the negatively charged oxygen atoms on the phosphate (Millan, 2006; Stec et al., 2000). Due to this conservation in the active site region, it has been proposed that the suggested reaction mechanism for the *E. coli* alkaline phosphatase (ECAP) applies to other APs as well. The proposed reaction mechanism is shown in Figure 1.1 (Coleman, 1992; Stec et al., 2000).

1.2 *Vibrio* alkaline phosphatase

The alkaline phosphatase studied herein is derived from the heat-labile bacterial strain *Vibrio splendidus* (VAP), which is a Gram negative marine bacterium closely related to *E. coli*. Similar to other APs, it is a homodimer composed of two loosely bound identical monomers and catalyzes the hydrolysis of phosphomonoesters. A ribbon structure of VAP is shown in Figure 1.2.
Figure 1.1 The catalytic mechanism of alkaline phosphatase. In the free enzyme (E), the phosphate-binding site is filled with three water molecules and the hydroxyl group of Ser102 forms a hydrogen bond with a Mg coordinated hydroxide ion. Upon substrate arrival, the ester oxygen on the substrate coordinates with the Zn1 while the other oxygen molecules interact with the Zn2 and the guanidinium group of Arg166 (Arg129 in VAP). This results in the formation of enzyme-substrate complex (EROP). Next the Mg-coordinated hydroxide ion acts as a general base and deprotonates Ser102, making it ready for a nucleophilic attack on the phosphorus atom. The result is an inversion of the phosphorus center, loss of the leaving group (RO\textsuperscript{-}) and consequently the formation of the covalent enzyme-phosphate intermediate (EP). The formation of the non-covalent enzyme-phosphate complex (EP\textsubscript{i}) is a result of an attack of the nucleophilic hydroxide ion coordinated to Zn1 and the subsequent second inversion of configuration at the phosphorus center. Finally, the Mg bound water molecule, facilitates the leaving of the inorganic phosphate from the non-covalent enzyme complex by acting as a general acid and donating a proton to O\textsuperscript{-} of Ser102 or, alternatively, inorganic phosphate. This leads to the reproduction of the free enzyme (E). Picture taken from (Stec et al., 2000).
While VAP has one of the highest reported catalytic activity for cold adapted alkaline phosphatases (APs), it also has the lowest reported thermal stability of known APs (half-life less than 10 min at 40 °C). Furthermore, its activity has proved to be very sensitive to urea, according to a recent study by Hjörleifsson & Ásgeirsson (2016), half of its activity is lost at only 0.3-0.4 M urea concentration. In the same study, which aim was to discover what factors are making VAP so susceptible to heat and urea, the unfolding pathway of VAP showed to be a three-step process taking place at 2-4 M urea concentration. The intermediates forming were believed to be inactive dimers (I$_2$), folded monomers (2I) and unfolded monomers (2U), as concluded by size exclusion chromatography results (N$_2$$\rightarrow$I$_2$$\rightarrow$2I$\rightarrow$2U). The fact that a formation of an inactive dimeric intermediate took place before the dimerization, supports the generally believed fact that VAP only is active as a homodimer. Regarding the instability of the enzyme, it is thought that it might be due to weakly bound Mg$^{2+}$ at the M3 site, the instability of the dimer or the combination of the two that the dimer association is cooperatively linked to Mg$^{2+}$ binding (Hjörleifsson & Asgeirsson, 2016).

VAP is thought to be a half sites reactive enzyme, meaning that the monomers take turns in binding a substrate molecule and catalyzing the reaction. This has however not yet been confirmed and the role of the dimer in catalysis has therefore not been established. However, a recent study by Kim et al. (2017) on the homodimeric enzyme fluoroacetate dehalogenase (FAcD) from *Rhodopseudomonas palustris*, provides a new insight into the possible role of the dimer in catalysis. The results of the study indicated that upon substrate binding to one monomer, the other monomer plays an important role of facilitating the reaction through enhanced dynamics by adopting greater short–time scale fluctuations and by shedding bound water molecules. In other words, the empty monomer has a role of sampling the subsequent functional states of the enzyme and compensating for the entropy loss associated with substrate binding (Kim et al., 2017). This could very well be the case for the VAP enzyme, however to determine it further studies are required.

There are many other unanswered questions regarding VAP, e.g. by what means the enzyme becomes inactive and whether there exists a dynamic equilibrium between the monomeric and dimeric form. Attempts to answer the latter are made in the study herein with the use of ion exchange chromatography.

### 1.3 Cold-adaptation

Cold ecosystems occupy over three-quarters of the Earth’s surface, including the oceans, alpine and polar regions. These areas have been successfully colonized by a class of microorganisms known as psychrophiles. Even where the temperature is permanently around or below the freezing point of water, these cold-living organisms have miraculously gained the ability to thrive (Feller & Gerday, 2003). What makes this possible is the vast array of adaptions through amino acid substitutions that bring in subtle changes in various enzymes at work (Asgeirsson & Andresson, 2001).
Figure 1.2 A ribbon representation of Vibrio alkaline phosphatase (PDB: 3E2D). The overall structural feature of Vibrio alkaline phosphatase is a central beta sheet running through the dimeric structure with helices packed on either site. The two monomers are colored red and blue and the catalytic zinc and magnesium ions, located in the active site of each monomer, are represented as gold and green spheres, respectively. Interactions between the crown domains of each monomer, located at the top of the figure, holds the subunits together. A noticeable loop (insert II) extends along the surface of the other monomer (red) contributing significantly to the dimer stabilization by the formation of several hydrogen bonds. Sulphates and ethylene glycol molecules are represented as ball-and-stick models, and originate from the medium (Helland et al., 2009).

Cold-adaptation is a particularly interesting phenomenon considering that the majority of enzymes in the biosphere work at low temperatures and dwell in permanently cold environments (Papaleo et al., 2013). For psychrophilic organisms, the evolutionary pressure is always at work improving intrinsic rate constants of enzymes in order to compensate for the effects that low temperatures have on the rate of physiological processes.

Many cold adapted enzymes from a range of organisms living in permanently cold conditions have been isolated and characterized (Feller & Gerday, 2003). The general characterization of cold-adapted enzymes is enhanced structural flexibility, reduced thermal stability and higher catalytic activity at low temperatures compared to their homologs from mesophilic organisms (Asgeirsson & Andresson, 2001; Papaleo et al., 2013). Other factors frequently associated with cold-adaptation are fewer salt bridges, fewer hydrogen bonds, extended surface loops and fewer prolines in loops (Jonsdottir et al., 2014; Siddiqui & Cavicchioli, 2006). The subunits in cold-adapted enzymes are in
general held together by weaker interactions than in mesophilic variants (Helland et al., 2009) and the weaker subunit interactions have previously been suggested to play a key role in cold-adaptation of AP (Papaleo et al., 2013). The reduction in subunit interaction may explain part of their improved catalytic action through increased mobility (Helland et al., 2009). Although the alkaline phosphatase from Vibrio splendidus (VAP) studied here has indeed a weak association of its dimers, it is the increased loop flexibility around the active site, one of the main characteristics of VAP, that may be contributing to the increased catalytic activity by holding the two subunits together while other interactions between them are reduced (Hjorleifsson & Asgeirsson, 2016). The other main characteristic of VAP is the large crown domain. Together those main features may very well contribute to minimizing the structural inertia that cold environments favour (Helland et al., 2009).

### 1.4 Historical beginnings of transverse urea gradient gel electrophoresis

Electrophoresis through polyacrylamide gels is one of the most commonly used methods for characterizing protein molecules. It is primarily a comparative technique in which only protein migration with time can readily be interpreted as a function of size (hydrodynamic volume/Stoke's radius) and net charge. Incorporating a gradient in the gels can allow for a particularly useful comparison of the electrophoretic mobility of different protein conformations under continuously varying conditions. Many types of gradients can be incorporated e.g. acrylamide, pH, urea and temperature (Goldenberg, 1989).

The transverse urea gradient gel electrophoresis (TUG-GE) method was first introduced by Thomas E. Creighton in 1979. Previously, in 1978, Creighton had discovered that the TUG-GE method was sensitive enough to distinguish between the compactness of trapped intermediates in protein folding and thus capable to characterizing their conformational properties. Until the TUG-GE method came to light, many uncertainties about protein folding transitions had remained unsolved, mainly due to technical limitations in studying protein conformation in solution. One particularly informative parameter that had not been fully utilized due to lack of suitable technique was the size and shape of the polypeptide chains in proteins. This physical property, however, differs markedly in folded and unfolded proteins and is, therefore, very convenient for the study of the unfolding/refolding processes of proteins.

When the TUG-GE method came to light, it seemed a very convenient method to analyze and answer the many unsolved questions about protein folding transitions, all on the basis of alterations of protein’s size and shape during the process of unfolding/refolding (Creighton, 1979). Fully folded proteins have a low hydrodynamic volume due to their compact structure and globular fold and that partly unfolded and fully unfolded proteins have larger hydrodynamic volume due to the loosening of the native fold (Siddiqui et al.). The key behind the method was the subsequent alteration of the electrophoretic migration of proteins as they would unfold along the increasing urea gradient in the polyacrylamide gels. Creighton ensured that the alterations in electrophoretic mobility due to the protein unfolding were primarily because of changes in the shape, or molecular volume of the peptide chains. However, another factor displayed effects on the electrophoresis mobility, namely the net charge of the protein.
Experiments that were performed changing the exposure of ionizable groups or bound ions, were proven to affect the electrophoretic mobility.

Indeed, after the TUG-GE method had been discovered, it proved to be a robust technique, capable of detecting various molecular fractions forming during unfolding/refolding of proteins (Creighton, 1979). Furthermore, because it is both a relatively simple and inexpensive method, it is ideal for characterizing the conformational properties of a protein as well as comparing the conformational stabilities of proteins since different proteins can be examined simultaneously (Creighton, 1979; Kalnine & Schachman, 2002; Siddiqui et al.).

1.5 Capabilities of TUG-GE and critical parameters

1.5.1 Capabilities, advantages and limitations

Transverse urea gradient gel electrophoresis is a simple method that allows for both quantitative and qualitative measurements (Goldenberg, 2001). The essence of the method is the slab gels which are prepared with a linear gradient of urea concentration perpendicular to the direction of the electrophoresis. That way, the protein band subjected to the electrophoresis, is faced with a continuous, linear, urea gradient (Goldenberg, 2001). As the urea concentration in the gel increases, more water molecules get associated with the urea. The result is a ruptured water structure and the creation of cavities in the bulk water. The effects of this decreased water activity along with the cavity formation causes increased solubility of hydrophobic side chains of the protein/s analyzed with concomitant protein unfolding (Siddiqui et al.).

The method has often been used to monitor denaturant-induced unfolding of proteins and ranks as one of the most convenient and inexpensive methods of those capable of selectively detecting chemically identical but conformationally different molecules (Kalnine & Schachman, 2002). However, for the TUG-GE to be useful, the protein analyzed must unfold/refold, reversibly or irreversibly, on the time-scale of the electrophoresis (Siddiqui et al.). Apart from allowing the direct analysis of the different structural and conformational states a given protein goes through, the method can also be used to estimate the free energy change and the rate of protein unfolding processes (D. P. Goldenberg, 2001). It also provides the potential of rapidly determine the kinetic and/or conformational stability of proteins under various conditions regarding the pH level, temperature or the usage of additives (Siddiqui et al.). Furthermore, since only the conformational stability and the effective size of a protein changes significantly over the transverse urea gradient due to the unfolding promoted by urea, the technique is particularly useful for comparing two forms of a protein (D. P. Goldenberg, 2001).

Since transient kinetic intermediates are undetectable in equilibrium experiments, the potential ability of detecting and identifying quantitatively these intermediates, is perhaps one of the main advantage of the TUG-GE method (Kalnine & Schachman, 2002). However, TUG-GE analysis has a couple of other great advantages e.g. its high resolution (Creighton, 1979), the small amount of protein required (about 0.1 - 10 µg are enough), the minimum effort needed to carry out the experiment and the compatibility with direct activity analysis (Siddiqui et al.).
A couple of inconveniences accompany the method as well as limitations. To start with the technical drawbacks, the method is not compatible with folding transitions involving more than one component (e.g. more than one polypeptide chain, cofactors, etc.) unless they can be incorporated into the gel. Also, only urea, alcohol and other uncharged denaturants can be used due to the interference the electrolytes that accompany charged denaturants (e.g. guanidine hydrochloride) cause in the electrophoresis. Finally, the environment of which the folding transition takes place is restricted to the environment of the electrophoresis (e.g. low ionic strength and pH levels that are not close to the pI of the protein) and the requirements that the polymerization of the acrylamide demands (e.g. no reducing agents). Turning to the disadvantages of TUG-GE analysis, the most apparent one must be the fact that the method gives no information on what type of conformation might be present. This can create a difficulty reading the results from the two dimensional patterns. Finally, there is always the risk of reaction with cyanate with the proteins (Creighton, 1979).

1.5.2 Critical parameters

More parameters have to be considered when TUG-GE is to be performed than e.g. SDS gel electrophoresis. The reason being that the behaviour of proteins during the TUG-GE depends on several properties of the particular protein that need to be controlled. These properties include the hydrodynamic volume, net charge and the conformational stability of the protein. Parameters that effect these properties include pH, gel composition and duration of the electrophoresis (D. P. Goldenberg, 2001).

The gel composition, i.e. type of urea gradient used, depends on the expected stability of the analyzed protein. TUG-gels are typically prepared with urea gradient from 0-8 M urea. However, the range can be altered. In general, thermolabile (cold-adapted) proteins require a lower urea range than thermophilic proteins (D. P. Goldenberg, 2001; Siddiqui et al.) To compensate for the inevitable decrease in electrophoretic mobility of proteins exposed to the higher concentrations of urea, it is possible to superimpose on the urea gradient an inverse gradient of acrylamide concentration (Goldenberg, 1989).

A suitable environment in terms of pH and temperature should be created for the protein analyzed to result in a successful TUG-GE. Thus, all solutions used must contain a buffer of an appropriate concentration and pH. Generally, if the protein analyzed is a basic protein (pI > 7) the pH of the buffer should be less than the pI of the protein by at least one pH unit, usually providing 5-10 charge units, and the electrophoresis should be carried out towards the positive electrode (anode). The opposite goes for acidic proteins (pI < 7). Sometimes it is necessary to use a pH far from the physiological pH e.g. to maintain a large enough net charge on the protein or to keep it soluble. In addition, the pH can be used to manipulate the stability of the protein. The gel temperature for the electrophoresis depends upon the sample analyzed. For psychrophilic proteins the gel apparatus can be placed in a refrigerator or a refrigerated room while electrophoresing (D. P. Goldenberg, 2001).

The gels are run for a suitable amount of time depending on the type and stability of the protein and its folding/unfolding behaviour (Siddiqui et al.). It also depends in the voltage applied to the gel and the type of information desired (D. P. Goldenberg, 2001). The two-dimensional pattern acquired after the electrophoresis gives a measure of the number of species present at a given urea concentration whether, be it a result from
heterogeneity of the protein or transient intermediates of the unfolding/refolding process (Creighton, 1979). The unfolding of a protein is detected as a decrease in electrophoretic mobility due to the larger hydrodynamic volume of the unfolded protein (D. P. Goldenberg, 2001).

1.6 Analysis of TUG-GE results

One of the greatest features of TUG-GE is the sensitivity it has towards the separation of various conformational states of proteins as well as the analysis of their thermodynamic and kinetic properties. By separating folded and unfolded protein molecules it is possible to observe the unfolding/folding transitions and measure the kinetic and conformational (thermodynamic) stabilities of proteins, as well as changes in activity. It can be difficult to assess with great certainty the various transitional events that occur to proteins (Siddiqui et al.). However, the general occurrence for protein unfolding in TUG-gels is the decrease in electrophoretic mobility due to the increase of the hydrodynamic volume of the protein throughout the unfolding process (Goldenberg, 1989). The diagram shown in Figure 1.3 exhibits schematically the general unfolding pattern obtained of a protein undergoing rapid unfolding process.

![Figure 1.3 A schematic representation of the unfolding curve of a protein undergoing rapid interconversion. The unfolding pathway produced by a urea gradient gel electrophoresis of a protein undergoing rapid interconversion between the native and unfolded states. The folded state exhibits a greater electrophoretic migration compared to the unfolded state due to the increased hydrodynamic volume of the unfolded state.](image)

Furthermore, the kinetics of the unfolding process is reflected in the continuity of the protein band. If the folding/unfolding equilibrium is rapid, compared with the time of the electrophoresis, a sharp continuous band is seen at the transition region (see Figure 1.3). However, if the equilibrium is slow, a discontinuous or smeared band will be detected (Goldenberg, 1989). Finally, rates of unfolding tend to increase and rates of folding tend to decrease with increasing urea concentration. Consequently, the folding/unfolding process is often relatively slow at the transition region while being very slow in the case of unfolding at low urea concentration and very slow in the case of folding at high urea concentration (Goldenberg, 1989). A graphical summary of the
unfolding and folding profiles of proteins as they would appear in TUG-gels is given in Figure 1.4.

(a) Rapid two-state
(F \xrightarrow{\text{fast}} U)

(b) Heterogeneous
(F \xrightarrow{\text{fast}} U_1
(F_2 \xrightarrow{\text{fast}} U_2)

(c) 2 forms of U
U_s \xrightarrow{\text{slow}} U_f \xrightarrow{\text{fast}} F

(d) Slow two-state
(F \xrightarrow{\text{slow}} U)

(e) Intermediates in unfolding
F \xrightarrow{\text{fast}} I \xrightarrow{\text{slow}} U

(f) Intermediates in refolding
U \xrightarrow{\text{fast}} I \xrightarrow{\text{slow}} F

(g) Rapid sequential unfolding/refolding
D_1D_2 \xrightarrow{\text{fast}} D_1 \xrightarrow{\text{fast}} D_2

(h) Slow\rightarrow rapid sequential unfolding/refolding
D_1D_2 \xrightarrow{\text{slow}} D_1 \xrightarrow{\text{fast}} D_2

Folded (F)  Unfolded (U)
**Figure 1.4 Schematic diagrams of unfolding/refolding patterns expected for monomeric and oligomeric proteins.** The pictures on the left represent the resulting patterns if a fully folded (F) protein is applied to the gel. The pictures on the right represent the resulting patterns if fully unfolded protein (U) is applied. For each sample, the direction of the electrophoresis is from top to bottom and the urea concentration increases from left to right. The fast (f) or slow (s) rates of unfolding/refolding of domain/s (D) refer to the rate constant for each transition which is the sum of the individual rate constants in the forward and reverse directions relative to the timescale of the electrophoresis (Siddiqui et al.).

If a protein has a high conformational stability, or if it has different conformations that inter-convert rapidly (i.e. exchange several times between folded and unfolded forms) compared to the duration of the electrophoresis, the result is a sharp and continuous band between the folded and unfolded conformation. If the protein furthermore follows a reversible mechanism of unfolding, that is if the pattern obtained for the unfolding is identical to that of folding, it is said to follow a two-state reversible mechanism of unfolding (Figure 1.4 a) (Siddiqui et al.). This is perhaps the simplest pattern observed, at low urea concentrations. The native form predominates while at high urea concentrations, the unfolded form predominates. At intermediate urea conditions both the forms are present at significant levels and the apparent electrophoretic mobility is the average mobility of the two forms, weighted by their relative abundance (Goldenberg, 1989).

In case of heterogeneous proteins (i.e. proteins that convey structural or conformational heterogeneity), the protein curves will split in the transition region (Figure 1.4 b). If a protein unfolds into two or more unfolded states, an observation like shown in Figure 1.4 c can be noticed. Scheme c depicts two populations of a preliminary unfolded protein of which one refolds rapidly (Uf) and the other one slowly (Us). In the extreme case illustrated here, none of the slowly folding form refolds and, therefore, a continuous straight band is seen.

The rate of both unfolding and refolding of any multi-domain proteins depends strongly on the urea concentration. Therefore, the same transition will be obtained whether a folded protein is put on the gel or the unfolded form (Figure 1.4 d). The only difference is that the unfolding of the folded protein occurs very rapidly at high urea concentration while the refolding of the unfolded protein occurs very fast at low urea concentration. Because the transition rate is much slower, in both the cases, at intermediate urea concentration, the protein states will be represented by a diffuse band (Figure 1.4 d) (Siddiqui et al.).

Figure 1.4 e and Figure 1.4 f show that kinetic intermediates of unfolding/folding can be detectable if one of the steps in the mechanism is slow. If the unfolded form slowly interconverts to an intermediate form that rapidly converts to or from the native state, then the intermediate is only detectible during unfolding (if the native form of the protein is applied), not during refolding (Figure 1.4 e). However, if the unfolded form rapidly interconverts with an intermediate that only slowly converts to or from the native form, then the intermediate is only detectable during refolding (Figure 1.4 f) (Goldenberg, 1989).
Sometimes, larger multi-domain proteins unfold independently at different urea concentrations, which results in a multi-step sequential unfolding shown in Figure 1.4 g and Figure 1.4 h. If all domains unfold/refold rapidly, within the duration of the electrophoresis, the result is a sharp and continuous curve throughout the gel as shown in Figure 1.4 g. If, however, one domain unfolds/refolds slowly at low urea concentration, a discontinuous curve is obtained like the one in Figure 1.34 h (Siddiqui et al.).

1.7 The aim of the project

For the past 20 years, the properties of the VAP enzyme regarding its catalytic activity in cold temperatures has been studied (Asgeirsson & Andresson, 2001; Heidarsson et al., 2009; Helland et al., 2009; Papaleo et al., 2013). What has been discovered is that the enzyme is active only in its dimeric form and that metal ions play a key role in maintaining the dimeric form and hence the enzyme’s activity. However, what remains to be understood, due to lack of suitable experimental methods, is the interplay and cooperativity of the monomers during enzyme catalysis.

Herein, a convenient method for assessing the monomer-dimer exchange was introduced, namely TUG-GE. This method is not only suitable for analyzing the unfolding/refolding process of protein but also analysing the kinetics of the process. We used it here to analyse the kinetics regarding the monomer-dimer exchange of two VAP variables at low urea concentration and the unfolding process at higher urea concentration. The hypothesis was that the strength of the monomer association in different VAP variants (where the primary structure has been changed) is often slightly altered, and that the difference is partly based on how tightly the magnesium ion in the reaction site is bound. The primary aim was set up the equipment needed to perform transverse urea electrophoresis in the laboratory for the first time and to get a good overview of the unfolding process by analysing the unfolding process taking place as urea concentration was increased and hopefully confirm the previously suggested unfolding mechanism described by Hjörleifsson and Ásgeirsson (Hjörleifsson & Ásgeirsson, 2016).

The second aim was to confirm the existence of a monomer-dimer equilibrium, that would result in monomer fluctuations. For this, differently charged monomeric forms of VAP were mixed and the resulting dimers separated using a high-resolution Q-Sepharose ion-exchange chromatography column. The hypothesis was that as the enzyme is constantly fluctuating between the monomeric and dimeric state as a result of a monomer-dimer exchange equilibrium, some of the dimers would have intermediary charge between the two extremes observed for the homodimer variants of either type. The two different C-terminal VAP variants (StrepTag or FLAG) had been shown to be distinguishable in an ion exchange chromatography (Sigurðsson, 2016), but the possible formation of a hybrid enzyme had not been tested for before.
2 Materials and methods

2.1 Materials

Chemicals were generally obtained from Sigma-Aldrich (Schnelldorf, Germany) or Merck (Darmstadt, Germany). USB® 5-bromo-4 chloro-3-indolyl phosphate, disodium salt for activity staining was purchased from Affymetrix (Santa Clara, USA). HiTrap Q-Sepharose FF column from GE Healthcare Life Sciences (Chicago, USA). Bromophenol Blue was purchased from Pierce Chemical (Dallas, USA). Novex R 4-20% pre-casted Tris-Glycine Mini Protein Gels used for SDS gel electrophoresis were purchased from Invitrogen (Carlsbad, USA). PageRuler™ prestained protein ladder and 10× Tris-glycine SDS buffer were acquired from Thermo Scientific (Rockford, USA). Part of the Strep-tagged VAP (VAP-ST2) variant used was obtained from the laboratory fully purified and aliquoted by T. Pálmadóttir (2011). Another part of the VAP-ST2 variant used and all of the 2x FLAG tagged VAP variant used were obtained from the laboratory fully purified and aliquoted by J. G. Hjörleifsson (2016). Still another part of the VAP-ST2 variant used was obtained from J. G. Hjörleifsson unpurified.

2.2 Protein purification

2.2.1 Purification of Vibrio alkaline phosphatase

Generally, all of the protein used was obtained from the laboratory fully rinsed and aliquoted by Jens G. Hjörleifsson (2016) and T. Pálmadóttir (2011). However, a part of the Strep-tagged VAP variant was not previously purified although it had previously been produced by Jens G. Hjörleifsson.

The Strep-tag is a short peptide sequence comprised of eight amino acids: Trp-Ser-His-Pro-Gln-Phe-Glu-Lys. Since this sequence has high affinity for streptavidin molecules and binds reversibly to them (Heidarsson et al., 2009), a Strep-Tactin® Sepharose® column composed of optimized streptavidin molecules immobilized on a Sepharose support, was used for the purification process.

The purification was performed at 4°C. A cell culture sample containing VAP with the Strep-tag purification tag (VAP-ST2) was generously provided by Jens G. Hjörleifsson (Hjörleifsson & Asgeirsson, 2016) as mentioned before. The sample had previously passed through a Strep-Tactin® Sepharose® column due to overloading and was reloaded onto the same column. The column was washed with TMC buffer (20 mM Tris, 10 mM MgCl2, pH 8.0) containing 500 NaCl and then TMC without any salt in order to minimize the presence of salt in the eluted protein sample and to remove all cell proteins other than bound VAP-ST2.

While washing, the absorbance at 280 nm was monitored. When the absorbance became insignificant the elution buffer (TMC buffer, 2.5 mM d-desethiobiotin, 15% ethylene glycol, pH 8.0) was loaded onto the column. The flow rate was set to 1.2 mL/min and the flow-through was then collected in Eppendorf tubes in 1 mL portions. The enzyme activity was measured for each and every one of the portions which subsequently were pooled together in two portions according to activity at 405 nm (see section 2.4). One of the portion consisted of samples showing high activity, and the other portion of samples

27
which showed low activity. The protein concentration in the two batches was then measured at 620 nm (see section 2.3).

### 2.2.2 SDS-PAGE protein electrophoresis

In order to assess the purity of the protein an SDS-PAGE protein electrophoresis was performed. The samples made were composed of 10 µl of NuPage (4x) LDS sample buffer and 30 µl of protein sample. To denature the proteins, the samples were incubated at 90°C for 5 minutes. Before they were loaded on the gel, they were cooled down at RT. The gel used was a Novex® 4-20% pre-casted Tris-Glycine Mini Protein Gel. It was loaded into a XCell SureLock™ Mini-Cell to which ca. 400 mL of Tris-glycine SDS buffer (0.025 M Tris, 0.195 M glycine and 0.1% SDS) were added (to both the lower and the upper buffer chamber).

The samples, 15 µL, as well as the protein ladder (PageRuler™ Prestained Protein Ladder from Thermo Scientific) were loaded into the wells of the gel with a syringe. After the loading of the samples the electrophoresis was begun. The voltage was set to about 160 V and the samples were electrophoresis for about 40 minutes. The gel was stained over night with Coomassie Brilliant Blue G-250 dye and de-stained the following morning by washing it in a 30% methanol solution.

### 2.3 Determination of protein concentration

The protein concentration of the purified VAP-ST2 portions (see section 2.2.1) was measured using a modification of the Bradford protein assay as described by Zaman and Verwilghen (1979). The method is based on the binding of proteins to Coomassie Brilliant Blue G-250, and the subsequent light absorption of the dye bound to the proteins. Each pooled sample was measured in triplicate using a spectrometer (Evolution™ 220, ThermoScientific) set to measure the light absorption at 620 nm. The samples were prepared by mixing 2.75 mL of Coomassie Brilliant-Blue G-250, 150 µL of deionized H₂O and 100 µL of purified enzyme solution in a glass test tube. The spectrometer was blanked, using a blank consisting of 2.75mL of Coomassie Brilliant Blue G 250 and 250 µL of deionized H₂O. The absorption of the samples was then measured in 3 mL plastic cuvettes.

In order to calculate the protein concentration from the absorbance results a standard curve previously prepared was used (y = 0.0056x, where y is the absorbance and x is the protein amount in µg).

### 2.4 Activity measurements

The activity of the VAP samples was measured at 25 °C using 5 mM p-nitrophenyl phosphate (pNPP) as a substrate and a 1.0 M diethanolamine buffer (1.0 mM MgCl₂, pH 9.8). As the enzyme catalyzes the dephosphorylation (hydrolysis) or trans-phosphorylation (in the presence of a suitable acceptor alcohol) of the pNPP substrate, the accumulation of p-nitrophenol and the subsequent yellow color of the solution is detected as a change in absorbance over a period of time. Meanwhile the diethanolamine in the buffer acts as a phosphate acceptor.
Prior to the enzyme assay, the protein samples were diluted 100x with the TMC buffer, if needed, and the spectrometer was blanked with the substrate (5 mM pNPP). The samples were prepared one at a time by pipetting 10 µL of the protein sample into a cuvette while the substrate was kept in a 25°C warm water bath. Then 990 µL of the substrate were added to the cuvette, which was then placed in the spectrometer and a measurement taken. The rate absorbance at 405 nm was measured for 30 seconds. By inserting the factor 5.405 into the spectrometer the activity was calculated as enzyme units per millilitre (U/mL) according to Beer’s law:

\[ A = \varepsilon \cdot l \cdot c \]

Where \( A \) is the absorbance, \( \varepsilon \) is the molar extinction coefficient (L mol\(^{-1}\) cm\(^{-1}\)), \( l \) is the path length of the cuvette and \( c \) is the concentration of the solution (mol L\(^{-1}\)). The 5.405 factor was calculated using the molar extinction coefficient of pNPP (18500 M\(^{-1}\) cm\(^{-1}\) at pH 9.8). The dilution of the protein samples in the beginning (10 µl sample / 1000 µl total volume) was accounted for in calculations of the final outcome.

2.5 Experimental Set-up for TUG-GE

2.5.1 Building of the experimental setup for TUG-GE

The protocol of Goldberg et al. (2001) was followed using parts of older electrophoresis systems available in our laboratory. The experimental requirements for TUG-GE consist mainly of a standard vertical gel-electrophoresis unit and a gradient mixer. Because it is not possible to buy a custom-made equipment for the TUG-GE method, one has to adapt equipment from other setups to use.

Instead of building the apparatus around a BioRad system, we used a gel casting block from Hoefer and adapted 0.75 mm spacers for transverse position of the glass plates during polymerization. An electrophoresis chamber from Invitrogen was used. A photograph of this initial setup is shown in Appendix Figure A2. To begin with, glass plates that were produced for various Hoefer gel electrophoresis systems were used. However, later on it was noticed that the width of the plate sandwich was marginal for the Invitrogen box used for the electrophoresis. Therefore, wider plates were made to solve this problem, 9 x 9 cm and 10 x 9 cm (H x W). Another problem came to light when the first gel was ready to run, namely a gap where the spacer runs alongside the larger plate only at the top. Goldberg et al. (2001) had a special spacer made to solve this problem. However, we managed to fit a small piece of a wider spacer (1 mm) with silicon grease to plug the gap.

Since the plugging of the gap was a troublesome work and not always working, another gel casting block was tried out. Instead of using the gel casting block from Hoefer and adapting 0.75 mm spacers, a homemade gel casting block and 1.5 mm spacers were used. Accompanying this, an electrophoresis chamber from Hoefer was used. A photograph of this setup is shown in Appendix Figure A3. This system worked well and eliminated the problem of leakage as well as making the gels thicker and more feasible to work with.
2.5.2 Setup of the gel casting apparatus

The protocol of Goldberg et al. (2001) was followed. First of all, three gel sandwiches were made using two glass plates, one rectangular and one with a slight cut-out on top forming two "ears" (9 x 9 cm and 10 x 9 cm H x W), and three 1.5 mm spacers for each sandwich. Two of the spacers were placed on the edges of each site between the glass plates while the third (shorter) was placed at the top on the curved glass plate, perpendicular to the other spacers to create a well for samples. Thin plastic films were placed between each of the sandwiches before they were stacked together in the casting block to avoid them sticking together after polymerization. The sandwiches were then placed in the casting box so that the spacer protected sides were aligned at the top and the bottom of the box. Before the casting block was closed, vaseline was put on the edges of the block to ensure no leakage. To prevent any leakage from the inlet of the block where the tubing from the peristaltic pump goes, a small piece of Teflon was wrapped around the ending of the tube.

Apart from the peristaltic pump, the casting block and the glass sandwiches the gel casting setup consists of a mixing chamber placed upon a magnetic stirrer. Two of the channels of the peristaltic pump, pump solution from this chamber during the gel casting process. In addition, there is a separate container for the 8 M urea solution, which the third channel of the peristaltic pump pumps from. Finally, a clamp is also needed during the preparation of the gel casting to clamp one of the channels containing 8 M urea solution to prevent backflow of the solution.

2.6 Preparation of the gel solutions

Stock solutions (0.5 L) were prepared of 0 and 8 M urea gel solutions. The ingredients in each solution are listed in Table A1. The gels had a photoactivating polymerization catalyst to allow sufficient time to prepare the gradient. The amount of TEMED needed to initiate polymerization changed proportionally with the increased volume of the gel solutions being 72 µl for each 40 mL portions of the gel solutions. A couple of gels were prepared containing 10 mM DTT which was then added and blended to the solutions before the degassing process took place (see section 2.7). Later on in the experiments, a couple of gels were made lacking the photoactivating polymerization catalyst, instead they contained ammonium persulfate. The ingredients for those gel solutions are listed in Table A2.

Two problems came up regarding the first 8 M urea stock solution made. The first problem being urea precipitation whilst degassing the solution, probably due to the cooling that followed the process. To prevent this problem, the 8 M urea solution was thereafter placed in a hot water-bath while degassing. This problem though seemed to disappear when a new solution was made and used. A new solution had to be made because of the second problem that occurred with the first solution, namely polymerization of the solution inside the stock solution bottle in advance upon storage (probably due to high acrylamide concentration and exposure to light). The main reason for the in advance polymerization was that the bottles used for storage were not made of amber glass and therefore not adequate for light sensitive solutions. Thereafter, the new solutions made were kept in amber glass bottles and away from light exposure.
When a third batch of the gel stock solutions were made, yet another obstacle came to light. The solutions proved to get more viscous much faster than before. In an attempt to tackle this obstacle, aluminium foil was wrapped around every container containing the solutions after the addition of TEMED to minimum the exposure to any light source.

2.7 Casting of the urea gradient gels

2.7.1 Casting of the transverse urea gradient gels (TUG-gels)

The casting of the gels were performed under subdued lighting. Various amounts of each solution were used to cast the gels depending on the size of the casting box. A couple of experimental runs were needed to assess the exact volume of solutions needed to fill the casting box and to determine a convenient ratio of how much of each solution should be placed in the mixing chamber and how much should be pumped in separately to create a convenient urea gradient.

For the Hoefer casting box used in the first gel casting processes, 20 mL of each solution were used plus an extra 0 M urea solution to fill the box completely (up to 10-15 ml extra). For the new homemade casting block, that was the ultimate apparatus used for the majority of the gel casting processes, 40 mL of each solution were needed to fill up the box. As mentioned earlier (see section 2.6) a couple of gels were made containing 10 mM DTT which was added to the solutions at this point (before the degassing process).

After measuring exact volumes of each solution, they were transferred into Büchner flasks equipped with a cap and degassed for 5 minutes with water aspirator. While degassing the 8 M urea solution bottle was kept in a warm water-bath due to previously mentioned problems.

During the degassing process, the peristaltic pump was pre-run with deionized water to prevent any blockage or bad elasticity of the tubes, the former resulting from a possible gel blockage from previous runs and the latter resulting from leaving the peristaltic pump closed overnight. After that, the TEMED was added to the solutions (72 µl to each 40 mL portion), right before the casting.

As mentioned in section 2.5.2, the setup of the gel casting also included a mixing chamber placed upon a magnetic stirrer from which two of the channels of the peristaltic pump pumped solution from. In addition, there was also a separate container for 8 M urea solution that one of the channels pumped from. All of the 8 M urea solution was placed in the 8 M urea solution container and a 5 mL Eppendorf pipette was used to fill the tubing connecting the reservoir to the mixing chamber. The tube was clamped (to prevent back-flow of the solution) and the outlet was temporarily placed over the 8 M urea solution reservoir. Next, 15 mL of the 0 M urea solution (various amount depending on the gel casting block used) was placed in the mixing chamber and the peristaltic pump was turned on to begin pumping the 0 M urea solution to the casting box at a rate of 2 mL/min (1 mL/min per channel). When the last of the solution had entered the tubing, the pump was stopped and the outlet of the 8 M urea solution tube opened and placed over the mixing chamber. The rest of the 0 M urea solution was added to the chamber (22.5 mL), then the magnetic stirrer was turned on as well as the peristaltic pump, now pumping a solution with linearly increasing urea concentration.
When the last of the solution entered the tubing, the pump was stopped again and the rest of the 8 M urea solution was placed in the chamber and pumped in. If the volume was not quite enough to fill the casting box, additional 8 M urea solution was added until the surface reached a sufficient height.

Next, the inlet to the casting box was clamped off, the box disconnected from the peristaltic pump and placed approximately 10 cm away from a lamp to initiate the polymerization which took at least 30 minutes. At first, the gels were stored in a refrigerator in a plastic bag with damp paper wrapped around them. However, due to a certain white precipitation that started to show up in the gels (likely urea) they were subsequently stored at RT which reduced the precipitation. A photograph of the final experimental setup for the preparation of the transverse urea gradient gels is shown in Appendix Figure A4.

2.7.2 Casting of the dual 0/8 M urea gels

Later in the experiments, a couple of gels were made where one half consisted of 0 M urea solution while the other half of 8 M urea solutions (dual gels). The protocol for the making of these gels is exactly as for the other TUG-gels except it is simpler. Instead of having to mix the 0 M and 8 M urea solutions in a gradient, half of the casting block was filled with 0 M urea solution using the peristaltic pump and then the 8 M urea solution was pumped in the block. Another way, perhaps more simple, for the making of these gels can also be done. That is, the 8 M urea solution can be poured directly into the casting block (after measuring the exact volume needed) and then the 0 M urea solution can be layered on top of the volume by using 5 mL Eppendorf pipette. The former method is more reliable since the same technique is used for the casting of TUG-gels (usage of a peristaltic pump), which has been tested by the addition of methyl orange dye to view the gradient formation.

2.8 Preparation and performance of urea gel electrophoresis

After the polymerization of the gels, the gels were rotated 90° from the orientation used for the casting process so the spacers were located on the top and the sites of the gels. By removing the spacer at the top of the gel, as oriented for electrophoresis, a sample well was created. Then the gels were assembled in the electrophoresis apparatus, one on each site. Two different electrophoresis buffers were used, at first 0.5 M Tris-acetate, pH 8.0 (0.5 M Tris base adjusted to pH 9.0 with acetic acid) was used. Later on another one was made, 0.5 M Tris/0.5 M Bicine (pH 8.4) or alternatively 0.5 M Tris/0.5 M Tricine (pH 8.4), with the hope of increasing the travelling distance of the protein on the gels. All chambers of the electrophoresis unit were filled up with the same buffer and the gels were pre-run generally at 30 mA (if 2 gels, otherwise 15 mA) for 30 minutes in order to remove any contaminates and to equilibrate the gels. The electrophoresis was performed towards the anode (positively charged).

During the pre-run the samples were prepared. Various amounts of proteins were used in each experiment, though most often 28 µg were used. The protein sample was then mixed with the basic protein sample buffer. Originally, the protocol of Goldenberg et al. was followed to mix the sample with basic protein sample buffer. However, the dye was
changed from being methyl green to bromophenol blue due to methyl green being positively charged (would not migrate towards the anode). The ingredients of the basic protein sample buffer and the electrophoresis buffer are listed in Table A3 and Table A4 respectively. Various ratios of protein sample to protein sample buffer were tested. Generally, a 1:1 (v/v) ratio seemed to work well. The samples were then loaded into the wells of the gels with a Finn pipette and distributed as evenly as possible over the well surface.

A couple of samples were denatured with urea before being loaded onto the gels. Either, 8 M urea solution was added to the protein sample until the final volume reached 6 M urea concentration or calculated amount of urea was weighted to the protein sample making the concentration of urea 6 M. To do so, the final volume had to be pre-estimated and the amount of urea calculated according to that. Deionized water was then added to the sample if needed until the final volume was reached. Next the samples were placed on a heat stand set to 25 °C and left there for 2-4 h which should be sufficient amount of time to denature the protein. To inhibit the refolding of the enzyme when entering the protein sample buffer, urea was also added to the sample buffer until the urea concentration reached 6 M using the same method as before.

The first electrophoreses were performed at room temperature (RT), but later on the electrophoresis chamber was placed in a refrigerator (4°C) due to the cold adaption of the protein analyzed and warming of the gels due to the flow of electric current. The time of the electrophoresis was variable while we tested for the best condition. At first, the electrophoresis was stopped after the carrier dye had reach the bottom of the gel. However, that was not enough time for the protein to migrate sufficiently far down the length of the gel. After many experiments, some of which extended overnight, a convenient amount of time was found, namely 5 hours. As before in the pre-runs, the current for each gel was set to ca. 15 mA.

After the electrophoresis, some of the gels were placed in activity staining buffer (1 M diethanolamine, 1 mM MgCl₂, pH 9.8) for about 20 minutes on a shaker. The they were submerged in an activity staining solution. Two different activity staining solutions were tried; one consisting of 2 mg of 5-bromo-4-chloro-3-indoyl phosphate disodium salt dissolved in 20 ml of activity staining buffer and another containing 6.7 mg/ml of naphthol-AS-MX phosphate and 1.0 mg/ml Fast red in 20 ml of activity staining buffer.

To visualize the gels, they were first submerged in a fixating solution (2.0% phosphoric acid and 30% ethanol) for 15 minutes. Next, they were put in water bath for 15 minutes two times. Thereafter, they were placed in a staining bath of either Coomassie R-250 or Silver blue (Coomassie G250) overnight. The day after, the gels were put in de-staining solution, which was deionized water for the silver blue gels and 25-30 % methanol solution for the gels colored with Coomassie R-250. A BioRad – GelDoc™ EZ Imager was used to photograph the gels.
2.9 Separation of VAP variants on a HiTrap QFF column

An elution gradient method in the form of a stepwise increase in ionic strength by increasing the NaCl concentration was created and used on a BioRad Logic pump apparatus in the cold-room at 4°C. It started with a 10 minutes wash with buffer A (20 mM Tris, 10 mM MgCl₂, pH 8.0) which was followed by a stepwise increase in the ratio of buffer B (20 mM Tris, 10 mM MgCl₂, 0.8 M NaCl, pH 8.0) to buffer A for 30 minutes until it reached 100% buffer B. Buffer B was on for 5 minutes before a stepwise decrease in the ratio of buffer B to buffer A took over which in 10 minutes reached 100% buffer A. In the end, buffer A was on for 5 minutes. Later on, the first step was elongated from 10 to 20 minutes of buffer A due to the volume size of one of the samples used. The flow rate was always set to 1 ml/min.

The VAP-ST2 variant samples were variably 0.12 - 0.54 mg and had the volume of 0.25 - 4.50 mL. No special preparation was needed before they were loaded onto the column. The 2x FLAG tagged VAP samples were variably 0.20 – 0.60 mg/ml and had the volume of 0.33 - 0.9 mL. Before the FLAG samples could be loaded onto the column they had to be centrifuged. The samples were placed in an Amicon centrifugal filter unit (YM30) and spun for 20 minutes at 4000 RCF (4°C) in a centrifuge (Hettich® Universal 320/320R centrifuge). Then, 1 mL of buffer A was added to the sample and the sample was spun again for another 20 minutes. In the end, the centrifugal filter unit was put upside down and spun for another 4 minutes to get all of the sample into the other end of the plastic collection unit. After that, the sample was ready to be loaded onto the column. The mixed samples, containing both variants (VAP-ST2 and 2x FLAG), were treated like the 2x FLAG-tagged VAP samples before they were loaded onto the column.

In the beginning of every run, the column was washed with buffer A for 5-10 minutes (flow rate 2 ml/min) to equilibrate the column. The sample was then loaded on the column with a syringe and the program started. The flow-through was most often collected in small test tubes (1 ml/min) and activity measured (see section 2.4).
3 Results

3.1 Purification of VAP-ST2 on Strep-Tag affinity column

A cell culture sample containing VAP with the Strep-tag purification tag (VAP-ST2) was generously provided by Jens G. Hjörleifsson (Hjorleifsson & Asgeirsson, 2016). The sample was purified with a Strep-Tactin® Sepharose® column. The purification table indicated a pure VAP with specific activity of 3000-3200 U/mg (Table 3.1).

Table 3.1 Protein purification table for the VAP-ST2 purified. The purification of the VAP-ST2 was carried out on a Strep-tactin Sepharose column. Protein concentration was measured using the Bradford protein assay. Activity was measured at 405 nm where enzyme was added to 5 mM p-nitrophenyl phosphate (pNPP) at 25 °C. Protein concentration was calculated from a standard curve with y (μg) = 0.0056x at 620 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume [mL]</th>
<th>Activity [U/mL]</th>
<th>Protein conc. [mg/mL]</th>
<th>Specific activity [U/mg]</th>
<th>Protein [mg]</th>
<th>Activity [U]</th>
<th>Yield [%]</th>
<th>Purification factor [x]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>140</td>
<td>831.5</td>
<td>2.18</td>
<td>381.4</td>
<td>305</td>
<td>116410</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Flow through</td>
<td>114</td>
<td>149.3</td>
<td>2.00</td>
<td>74.6</td>
<td>228</td>
<td>17020</td>
<td>14.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Elution I</td>
<td>7.75</td>
<td>722.7</td>
<td>0.24</td>
<td>301.1</td>
<td>1.9</td>
<td>5601</td>
<td>4.8</td>
<td>7.8</td>
</tr>
<tr>
<td>Elution II</td>
<td>5.9</td>
<td>301.5</td>
<td>0.093</td>
<td>3246</td>
<td>0.5</td>
<td>1778</td>
<td>1.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

3.2 Building of the apparatus and determination of ideal condition for TUG-GE

3.2.1 Building of the apparatus

The first attempt to mimic the apparatus in the protocol of Goldenberg et al. (2000), used parts of older electrophoresis systems available in our laboratory (Figure A1 in the Appendix). A gel casting block from Hoefer was used and 0.75 mm spacers were adapted to fit the glass plates for transverse position of them during the polymerization. The glass plates used were produced for various Hoefer gel electrophoresis systems.

During a test run of the peristaltic pump and the whole apparatus, a small leakage appeared in the inlet of the gel casting block. The leakage was taken care of by wrapping small amount of Teflon around the inlet tube.

Initially, an electrophoresis chamber from Invitrogen was used for the electrophoresis (Figure A1). While preparing a test electrophoresis run, we found out that the width of the plate sandwich was marginal for the Invitrogen box used for electrophoresis. Therefore, wider plates were made to solve this problem, 9 x 9 cm and 10 x 9 cm H x
W. Another problem came to light when the first gel was ready to run, namely a gap where the spacer runs alongside the larger plate only at the top. Goldberg et al. (2000) had a special spacer made to solve this, but we managed to fit a small piece of a wider spacer (1 mm) with silicon grease to plug the gap.

### 3.2.2 Determination of the ideal conditions for the TUG-GE of VAP

The conditions described in the protocol of Goldenberg et al. (Goldenberg, 2000) were used as a criteria for the determination of the conditions used here. We chose buffer conditions at pH 8.0 as the *Vibrio* alkaline phosphatase has a calculated isoelectric point of 5.4 ("ProtParam,"), so should be fully negatively charged at this pH. However, the isoelectric point has also been recorded to be as high as 7.6 using gel isoelectric focusing (Hauksson et al., 2000). The tracking dye used was bromophenol blue, which is different to the methyl green suggested by the protocol. We think the original authors reversed the composition of their "basic" and "acidic" samples buffer dye, since methyl green is a positively charged molecule and would not travel toward the cathode with the proteins. The first TUG-GE were performed at RT, however later on we switched to performing them at 4°C due to increased stability of the enzyme at lower temperatures.

### 3.3 The first electrophoreoses

#### 3.3.1 The first TUG-GE performed on native VAP

The first electrophoresis of VAP in the presence of urea was performed using the apparatus described in section 3.1.1 in order to adjust the amount of protein applied and the time of the run. The first sample (75 µl of 0.14 mg/ml) was electrophoresed at RT in 0.5 M Tris-acetate buffer, pH 8.0, for 2 h and 30 min with a current of 15 mA. The second sample (90 µl of 0.14 mg/ml) was electrophoresed at the same conditions as before except the time was 3 h and 20 min and the current was set to 10 mA. After staining with Coomassie Blue R-250, a clear band was observed traversing the gel toward the bottom of the gel in the former case, but close to the top of the gel in the second case. Unfortunately, these gels were lost before a picture could be taken, but they indicated that there were leaks in the home-made system allowing the current to bypass the gel, since the protein was travelling a very short distance into the gel.

Several attempts were then made to cast gels and improve the set-up, but some problems arose. Either the peristaltic pump got clogged, or the flow of the solution in the tubes was disrupted, creating a lot of air bubbles and a subsequent uncertainty about the quality of the urea gradient forming. During these trial runs, the volume of each gel solution and the blending process was developed.

Figure 3.1 shows the third run that was carried out with the same conditions as before, apart from a considerable increase in the amount of the phosphatase used (50 µl of 1.4 mg/ml). Also, to test if both bromophenol blue and methyl orange tracking dyes had the same electrophoretic mobility, both these dyes were used in the sample buffer.
As can be seen in Figure 3.1, two protein bands appeared at the top of the gel. Both bands had a slight curvature towards each end of the urea gradient, and one of the bands was much stronger than the other. Apparently, the additional time and the increased amount of protein gave clearer results. Somewhat surprisingly, the urea was not affecting the migration of these two bands much, although the bands were more diffuse in the low urea part. No further speculations were made regarding those results. Instead, the focus was turned to improving the electrophoresis conditions by changing the apparatus and performing more trial runs.

### 3.3.2 The first TUG-GE performed on denatured VAP

The last electrophoresis performed using 0.75 mm gels before turning to 1.5 mm gels was of urea denatured VAP. Twenty 20 µl of 1.4 mg/ml sample were used and denatured in 6 M urea solution at 25°C for 4 h. The ratio of protein to sample buffer was 1:1. The sample was supposed to be electrophoresed for 18-24 hours, in hope of increasing the migration distance of the protein. However, due to a leakage of the system, the electrophoresis was interrupted so the total run time was about 10 hours. Unfortunately, the gel was lost before a picture could be taken. However, the results showed that the protein band had migrated a bit further than in the previous runs. However, it was still on the upper part of the gel. It was somewhat surprising to see how slowly VAP travelled in the urea gels, but the distance observed by others using
different proteins is generally not easy to ascertain from figures since only a selected part of the gels is shown magnified.

### 3.4 Improvements of the apparatus

Because the electrophoresis using the modified system meant for running NuPage gels system was hampered by leakage of the electrophoresis chamber, a whole new TUG-GE setup was taken in use. Instead of using the gel casting block from Hoefer and adapting 0.75 mm spacers, from here on a homemade gel casting block and 1.5 mm spacers were used. Accompanying this, an electrophoresis chamber from Hoefer was used (Figure A3). It was also decided to use a more basic electrophoresis buffer (Tris-Bicine at pH 8.4 instead of pH 8.0) for the next TUG-GE runs, to ensure that the phosphatase was fully negatively charged (pI 4.8). This is necessary for it to migrate at maximum speed down the gel.

### 3.5 The first electrophoreses using the improved apparatus: controls

#### 3.5.1 Electrophoresis of α-amylase and myoglobin

Using the new thicker gels, two well-known proteins were electrophoresed, namely a bacterial amylase from *Bacillus subtilis*, α-amylase, and myoglobin from equine heart. Also, a more basic electrophoresis buffer was used, 0.5 M Tris/0.5 M Bicine (pH 8.4), instead of the 0.5 M Tris-acetate (pH 8.0), in hope of increased migration of the proteins. Both samples contained 50 µg of protein and 50 µl of sample buffer. The electrophoresis was run until the bromophenol blue dye reached the bottom of the gels (3-4 h). Figure 3.2 and 3.3 show the results. The proteins did not travel further down the gel than the VAP enzyme in the previous runs. However, the effect of urea was clear, i.e. the protein bands showed a distinct inclination throughout the increasing urea concentration in the gels.
Figure 3.2 TUG-GE of bacterial α-amylase from Bacillus subtilis performed at RT in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer. The direction of the electrophoresis is from top to bottom and the urea gradient (0-8 M) increases from left to right. Three protein bands are showing, the most intense line located at ca. 0-5 M urea concentration is probably the folded protein. The line to the right in the 8 M urea end could be the unfolded protein and the uppermost line could also be unfolded protein.

Figure 3.3 TUG-GE of myoglobin from equine heart. performed at RT in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer. The direction of the electrophoresis is from top to bottom and the urea gradient (0-8 M) increases from left to right.
3.5.2 Electrophoresis of native VAP at RT

In the hope of increased migration of VAP by increasing the current applied, two 28 µg VAP samples with a protein to sample buffer ratio of 1:4, were electrophoresed for 5 h with a current of 40 mA (20 mA each, a double from last runs). Activity staining was performed on one of the gels, while the other one was stained with Silver Blue (Coomassie G250). Two different activity staining solutions were tried; 2 mg of 5-bromo-4-chloro-3-indoyl phosphate disodium salt in 20 ml of activity staining buffer (1 M diethanolamine, 1 mM MgCl2, pH 9.8) and 6.7 mg/ml of naphthol-AS-MX phosphate with 1.0 mg/ml Fast red in 20 ml of activity staining buffer. Both resulted in negative results. At that time, the reason behind the inactivity was thought to be either due to excess heat formation during the electrophoresis or that the 0 M urea region in the gel was to small and, therefore, did not extent to the space where the native protein sample was loaded onto. It later turned out that riboflavin used to polymerize the gels is an inhibitor of VAP activity.

As can be seen in Figure 3.4 the migration of the protein increased. Compared to the first TUG-GE results of VAP (Figure 3.1). This experiment also resulted in two protein bands, the lower being much more intense than the other. The main difference is, however, the upward incline of the bands as the urea concentration becomes greater, and the enlarged curve near the 0 M region. The incline could be due to the unfolding of the dimer. The higher band might be due to unfolded monomers or precipitation of the enzyme due to formation of disulfide bonds, since it is not present in the lowest urea concentrations.

![Figure 3.4 TUG-GE of VAP performed at RT in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer. The sample contained 28 µg protein and the ratio of protein versus sample buffer was 1:4 (v/v). The sample was electrophorized for 5 hours and the current was set to 20 mA per gel. The dots on the gel are most likely urea precipitation, which did not seem to affect the electrophoresis at all.](image)
As can be seen (Figure 3.4), urea precipitation occurred in the gels. It is thought that the promotion of the precipitation might be small impurities that were left on the glass plates and also the storing of the gels in a refrigerator at 4°C.

### 3.6 TUG-GE performed at 4°C

#### 3.6.1 Electrophoreses of native VAP

Next we tested the reproducibility of the technique. Like in the first electrophoresis of VAP, this experiment was performed at 4°C and by using 28 µg of protein (40 µl) dissolved in 80 µl of protein sample buffer. Two electrophoresis runs were carried out simultaneously using two identical samples having the same amount of protein. The samples were electrophoresed as before in 0.5 M Tris/0.5 M Bicine, pH 8.0, for 5 h with a current of 37 mA. After the electrophoresis, one of the gels was activity stained using the same solutions as before. No activity was observed. Both of the gels were then fixed (2.0% phosphoric acid, 30% ethanol) and stained with Silver Blue overnight. The results were a sharp straight band with a distinct upward curvature at the low urea concentration region, (see Figure 3.5 and Figure 3.6).

![Image](image_url)

**Figure 3.5 TUG-GE of VAP performed at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer.** The sample contained 28 µg protein and the ratio of protein versus sample buffer was 1:2 (v/v). The sample was electrophorized for 5 hours and the current was set to 37 mA. The dots on the gel are most likely urea precipitation, which did not seem to affect the electrophoresis at all.
The curve could result from the beginning of the denaturation of the enzyme at low urea concentration, i.e. that the dimers are beginning to dissociate and unfold resulting in shorter migration (upward curve). The downward ending of the curve at 1.5-2 M urea could be due to the completeness of the dissociation before the monomers are completely unfolded, resulting in increased migration of the smaller polypeptide chains (the monomers). The curve ends with a straight continuous line which seems to have a small upward incline. The incline could be due to the continuous denaturation of the monomers and the subsequent increase in hydrodynamic volume as the urea concentration increases. The gels were not fully identical but indicated in common that unfolded VAP monomers at 8 M urea do not travel much differently compared with the initial sample at 0 M urea.

**Figure 3.6** TUG-GE of VAP performed at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer. The sample contained 28 µg protein and the ratio of protein versus sample buffer was 1:2 (v/v). The sample was electrophorized for 5 hours and the current was set to 37 mA. The dots on the gel are most likely urea precipitation, which did not seem to affect the electrophoresis at all.

This electrophoresis experiment was repeated with the same amount of protein and same conditions except the current was decreased from 37 mA to 30 mA per gel. Now, only one of the gels was stained with Silver Blue, while the other was activity stained. No activity was observed as before. However, the results from the protein staining were interesting (Figure 3.7).
Figure 3.7 TUG-GE of VAP performed at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer. The sample contained 28 µg protein and the ratio of protein versus sample buffer was 1:2 (v/v). The sample was electrophoresed for 5 hours and the current was set to 30 mA.

Three bands were observed, one of them having much greater intensity than the others. As a suggestion, one of the bands could be representing some sort of aggregated protein. Also, the band showing the greatest migration (the most intensive band) could be representing the formation of a molecular species having different net charge compared to the initial enzyme, possibly due to deamination.

3.6.2 Electrophoresis of urea denatured VAP

A VAP protein sample (28 µg) was denatured in 6 M urea solution for 2 h at 25°C. To maintain the urea concentration in the final sample, urea was added to the protein sample buffer until it reached 6 M urea concentration. The ratio of sample vs. sample buffer was 1:1. The sample was electrophoresed as before in 0.5 M Tris/0.5 M Bicine, pH 8.4, for 5 h with a current of 10 mA. The result is shown in Figure 3.8. As can be seen, two bands were observed, one having much greater intensity than the other. The more intense band is probably the unfolded protein while the weaker band is possibly aggregated protein. There was a slight upward incline in the bands toward the low urea concentration region, indicating the possibility that the protein had started to refold. The band travelling furthest in Figure 3.7 was missing (assuming equal travelling distances overall).
3.6.3 Electrophoresis of Strep-tagged and FLAG-tagged VAP on DTT containing TUG-gels

At this point in the project, only Strep-tagged VAP had been electrophoresed. Now, VAP-ST2 and 2x FLAG tagged VAP were simultaneously electrophoresed on TUG-gels containing 10 mM dithiothreitol (DTT). The expectation from the addition of DTT into the gels was to disrupt the disulfide bonds that possibly were forming and promoting the formation of aggregated protein through the single cysteine-thiol that each VAP monomer has. Both the samples contained 28 µg of protein and had a 1:1 ratio of protein vs. sample buffer. The electrophoresis was carried out in 0.5 M Tris/0.5 M Bicine buffer (pH 8.4), for 5 h with a current of 80 mA in the beginning (in attempt to increase the protein migration) which was shortly lowered to 40 mA (20 mA per gel).

The gel containing the VAP-ST2 sample was activity stained using the same activity solutions as before. However, no activity was observed. Later, it was stained in Silver Blue only to see that the protein had travelled all the way to the bottom of the gel (Figure A4). Unfortunately, it was not manageable to get the gel containing the 2x FLAG tagged VAP sample of the glass plate in one piece, since it was very stuck to the plate. Because it was shattered to many pieces, it was not activity stained. However, it was stained with Silver Blue. The result was the same as for the other gel. The protein band had travelled through the gel. Setting the current to 80 mA in the beginning of the electrophoresis is undoubtedly the reason for the great increase in the protein migration as was observed. Consequently, due to the high voltage, there was also a great heat formation during the electrophoresis which led to the gel sticking to the glass plate as much as it did.
The previous electrophoresis experiment was repeated with the same amount of protein and same conditions except the current was set to 35 mA (not 80 mA). Both the gels were fixed and stained with Silver Blue. The results are shown in Figures 3.9 and Figure 3.10.

Figure 3.9 TUG-GE of VAP-ST2 performed on a gel containing 10 mM DTT, at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer. The sample contained 28 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The sample was electrophoresed for 5 hours and the current was set to 35 mA.
Figure 3.10 TUG-GE of 2x FLAG tagged VAP performed on a gel containing 10 mM DTT, at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) buffer. The sample contained 28 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The sample was electrophorized for 5 hours and the current was set to 35 mA.

As can be seen in Figure 3.9, the electrophoresis of the VAP-ST2 sample resulted in only one intensive protein band. The band is unlike any other results acquired of the VAP-ST2 variant, as the protein band was curved all the way through the urea gradient. In the beginning, there is a great upward incline where the band is sort of smeared out. This could indicate a slow unfolding of the enzyme, as a smeared protein band indicates a slow unfolding/refolding transition whereas a sharp band indicates a fast transition (see section 1.6). At average urea concentration, the band is somewhat straight with a little upward incline all the way until a substantial higher urea concentration is reached, where a sharp downward curve appears. Upward incline might be due to unfolded monomers that are presumably of larger hydrodynamic volume than either the dimers or folded monomers. It would suggest that the folded monomers are present in the fuzzy area with the dimers.

As can be seen in Figure 3.10, the electrophoresis of the 2x FLAG tagged VAP sample resulted in three main protein band, one intensive and two weak. As many of the protein bands acquired with VAP-ST2 samples, the intensive band seen in Figure 3.10 has an upward curvature at low urea concentration below 2 M. At intermediate urea concentration, the band is somewhat straight with a small downward decline. At very
high urea concentrations a very sharp downward curve appears. The sharp curve at the 8 M urea end could be due to some artefact regarding the electrophoresis, since there was also a downward curve at this point in the gel containing the VAP-ST2 sample (see Figure 3.9).

The bands in the middle and top of the FLAP-VAP experiment of Figure 3.10 only started at intermediate urea concentration. Therefore, it could be representing the completeness of the dimer dissociation and the subsequent formation of monomers which fits recent studies on urea treated VAP (Hjorleifsson & Asgeirsson, 2016) takes place somewhere between 2-4 M urea concentration. It is not quite understood what the uppermost band could be representing. It is only observed at the higher end of the urea gradient, which indicates that it is an unfolded protein. However, because the gel contained 10 mM DTT, it is not likely that it is aggregated protein.

To have a comparison to the previous electrophoresis of the VAP-ST2 and 2x FLAG tagged VAP variants on gels containing 10 mM DTT, a bacterial amylase from Bacillus subtilis, α-amylase, was electrophoresed in the same conditions. The only variation from the other electrophoresis with DTT containing gels was the amount of protein used which was 50 µg. Because only one gel was electrophoresed, the current was set to 15 mA. The result was a smudged protein band situated on the lower part of the gel (Figure A5).

### 3.6.4 Electrophoresis of VAP on dual 0/8 M urea gels

In an attempt to achieve clearer results, different kind of urea gels were tried with no gradient. A couple of dual gels were made, where one half consisted of 0 M urea solution and the other half of 8 M urea solution with a sharp boundary. By using this kind of gels, it should be possible to determine the migration of the folded and unfolded form of the enzyme as well as facilitate the comparison between the two forms.

In the first experiment using a dual 0/8 M urea gel, two gels were run simultaneously. One was loaded with 14 µg of VAP-ST2 protein sample (117 µl) and the other with 14 µg of denatured VAP-ST2. To denature the sample, the protein was kept in a 4 M urea solution and heated to 50°C for 15 minutes. It should be mentioned that the denaturizing procedure could possibly have caused cyanate formation. In any case, urea was also added to the protein sample buffer, until a 4 M solution was reached. The protein sample was then dissolved in 1:1 ratio (v/v) of protein sample buffer. The electrophoresis was carried out in 0.5 M Tris/0.5 M Bicine-Tricine buffer (pH 8.4), for 5 h with a current of 30 mA. After the electrophoresis, both gels were submerged in activity staining buffer (1.0 M diethanolamine, 1.0 mM MgCl₂, pH 9.8) for 15 minutes and then activity stained by adding 2 mg/20 mL of 5-bromo-4-chloro-3-indoyl phosphate disodium salt. No activity was observed in the gels. Next they were stained with Silver Blue, the results can be seen in Figure 3.11 and Figure 3.12.
**Figure 3.11** Electrophoresis of native VAP-ST2 performed on a dual gel. One half of the gel consists of 0 M urea solution (left) and the other of 8 M urea solution (right). The electrophoresis was performed at 4°C in 0.5 M Tris/0.5 M Bicine-Tricine (pH 8.4) buffer with a current of 30 mA. The sample contained 14 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The sample was electrophorized for 5 hours.
Figure 3.12 Electrophoresis of denatured VAP-ST2 sample performed on a dual gel. One half of the gel consists of 0 M urea solution (left) and the other of 8 M urea solution (right). Prior to the electrophoresis the sample was kept in a 4 M urea solution and heated to 50°C for 15 minutes to denature the protein. Urea was also added to the protein sample buffer, until a 4 M solution was reached. The sample contained 14 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The electrophoresis was performed at 4°C in 0.5 M Tris/0.5 M Bicine-Tricine (pH 8.4) buffer with a current of 30 mA. The sample was electrophorized for 5 hours. The vertical lining seen throughout the protein band, is presumably some artifact of the electrophoresis.

The electrophoresis of the native VAP-ST2 sample gave a very interesting result, where all the three aforementioned forms of the enzyme were possibly witnessed, i.e. dimer, monomers and unfolded monomers. As can be seen in Figure 3.11 of the native VAP-ST2 sample, three protein bands appeared in the 0 M urea solution part of the gel, of which only one extended to the 8 M urea solution part. Therefore, it is assumed that the long band extending to both halves of the gel is the unfolded enzyme. Furthermore, this band is also more intense at the 8 M urea half and is only apparent nearby the center of the gel in the 0 M urea solution half, i.e. it fades out the longer it moves from the 8 M urea half. That supports the assumption that it represents unfolded enzyme, i.e. unfolded monomers. The weak band appearing slightly above the aforementioned band (in the 0 M half) could then very well be the folded enzyme (dimer) or folded monomers. It is possible that the part of the longer band extending to the 0 M urea part is representing folded monomers as this part of the gel very likely contains low urea concentration (sufficient enough to cause dimer dissociation) and the band is showing a small upward incline towards the center that could be due to the unfolding of the monomers encountering a higher urea concentration. The uppermost band located in the 0 M half could possibly be representing the folded enzyme (dimer) or some sort of aggregated/unfolded enzyme.
As can be seen in Figure 3.12, the electrophoresis of the denatured VAP-ST2 sample only resulted in a single weak and diffused band. A possible reason for the low intensity of the band is that some of the protein did not manage to migrate down and was sitting at the top of the gel (the dark band on top of the well). However, the protein band forms a horizontal line throughout the 0 M urea part and half of the 8 M urea part. Then, it takes on a small curvature. It is unclear what is causing this pattern. However, it is somewhat similar to the pattern observed in the 8 M urea part in Figure 3.11 (the curvatures have similar shapes). Notably, the unfolded form in 8 M urea was travelling the furthest in this experiment.

The most important outcome from this part of the experiment is the fact that it appears that all the enzyme was still in the unfolded form, i.e. none of the enzyme managed to refold during the electrophoresis. Finally, the vertical lining observed throughout the protein band, is presumably some artefact of the electrophoresis.

The electrophoresis of native VAP-ST2 on a dual urea gel was repeated exactly as before using the same amount of protein. The result is shown in Figure 3.13.

Figure 3.13 Electrophoresis of VAP-ST2 performed on a dual gel. One half of the gel consists of 0 M urea solution (left) and the other of 8 M urea solution (right). The electrophoresis was performed at 4°C in 0.5 M Tris/0.5 M Bicine-Tricine (pH 8.4) buffer with a current of 30 mA. The sample contained 14 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The sample was electrophorized for 5 hours.

As can be seen from Figure 3.13, the results were not at all similar to the ones gotten from the previous experiment (Figure 3.11). Only a single protein band was observed entering the gel, being most intense at the 0 M part of the gel and fading out in midst of the 8 M part. It is a possibility, due to the incline in the sample well of the gel, that the
sample could have flowed/leaked slowly to the other end of the gel (0 M urea), leaving nothing or a little amount of protein left at the other end (8 M urea), thus resulting in no protein band being seen at the 8 M urea end. Some of the sample remained on the top of the gel.

In the final experiment performed on dual urea gels, the simultaneous electrophoreses of two native VAP-ST2 samples differing only in the amount of protein used was performed in an attempt to assess if the amount of protein is affecting the resulting pattern. One weak and one strong sample were made containing, 12 and 40 µg of protein respectively. Each sample had a protein to sample buffer ratio of 4:10 (v/v), a bit lower than usual to increase the volume to the samples to facilitate an even distribution of the samples to the sample-wells. As before the electrophoresis was carried out in 0.5 M Tris/0.5 M Bicine-Tricine buffer (pH 8.4), for 5 h with a current of 30 mA. The results are shown in Figure 3.14 and Figure 3.15.

**Figure 3.14 Electrophoresis of VAP-ST2 performed on a dual gel.** One half of the gel consists of 0 M urea solution (left) and the other of 8 M urea solution (right). The electrophoresis was performed at 4°C in 0.5 M Tris/0.5 M Bicine-Tricine (pH 8.4) buffer with a current of 30 mA. The sample contained 12 µg protein and the ratio of protein versus sample buffer was 4:10 (v/v). The sample was electrophoresed for 5 hours.
Figure 3.15 Electrophoresis of VAP-ST2 performed on a dual gel. One half of the gel consists of 0 M urea solution (left) and the other of 8 M urea solution (right). The electrophoresis was performed at 4°C in 0.5 M Tris/0.5 M Bicine-Tricine (pH 8.4) buffer with a current of 30 mA. The sample contained 40 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The sample was electrophoresed for 5 hours.

As can be seen from Figure 3.14 and Figure 3.15, electrophoresis of the relatively small amount of protein (12 µg) resulted in only one protein band whereas the electrophoresis of the relatively large amount (40 µg) resulted in two protein bands. It can, therefore, be concluded from the comparison of these gels that having greater amount of protein increases the likelihood of seeing two or more protein bands instead of only one. Furthermore, having greater amount of protein could play a role in the unfolding process of the protein or the possible equilibrium between the monomeric and dimeric forms.

3.7 Timing of Strep-tagged and 2x FLAG tagged VAP elution on a Q-Sepharose column

In an attempt to determine if there exists a monomer-dimer equilibrium in VAP, which would result in a fluctuation between the monomer and dimer forms, a mixture of two VAP variants was applied to a strong anion exchange column. It was decided to use a Q-Sepharose Fast Flow (HiTrap™ 1 ml QFF) column on an FPLC apparatus (BioRad Logic). The hypothesis was that if there is an equilibrium, a third variant will appear in between the two variants applied to the column. Because the two variants used, VAP-
ST2 and 2x FLAG tagged VAP, are separable on the column on the basis of the differences in their net charge of 6 negative units per monomer, it should be possible to identify the third variant.

The samples were loaded on the column and eluted with 0-0.8 M NaCl gradient. At first, only one variant at a time was applied to the column to determine when they were eluted. The VAP-ST2 samples contained variably 0.12 - 0.54 mg of protein and had the volume of 0.25 mL - 4.50 mL. The 2x FLAG tagged VAP samples contained variably 0.20 – 0.60 mg/ml and had the volume of 0.33 - 0.9 mL. In Figure 3.16, two representative chromatographs of VAP-ST2 and 2x FLAG tagged VAP samples have been superimposed. As can be seen, the two variants can clearly be separated on the Q-Sepharose column. In Figure 3.17 and Figure 3.18, the two chromatographs of the samples have been plotted up against the salt gradient. As can be seen, the VAP-ST2 protein eluted at a salt concentration of about 28 mS/cm (ca. 39 min) while the 2x FLAG tagged VAP protein eluted a little later at a salt concentration of about 39 mS/cm (ca. 45 min). A noticeable shoulder peak is on the left side of the VAP-ST2 sample peak (Figure 3.17), it is not clear what it represents.

![Figure 3.16 The separation of Strep-tag and 2x FLAG tagged VAP on a Q-Sepharose column. The chromatograms are superimposed on each other. Time is on the x-axis while the absorption at 280 nm is on the y-axis. The Strep-Tag VAP sample is 0.54 mg while the 2x FLAG tagged VAP sample was 0.60 mg.](image-url)
Figure 3.17 A representative chromatograph of Strep-tagged VAP sample loaded on a Q-Sepharose column. Time is on the x-axis while both absorption at 280 nm and the salt gradient are on the y-axis (on either site). The Strep-Tag VAP sample is 0.54 mg.

Figure 3.18 A representative chromatograph of 2x FLAG tagged VAP sample loaded on a Q-Sepharose column. Time is on the x-axis while both absorption at 280 nm and the salt gradient are on the y-axis (on either site). The 2x FLAG tagged VAP sample is 0.60 mg.

The flow through of the column was collected in small test tubes (1 ml/min) in order to measure the enzyme activity and compare it to the elution peaks resulting from the Q-Sepharose column experiments. The enzyme activity of the samples used in Figure 3.16 are listed in Table A5 in Appendix. The activity measurements fitted very well with the
results in Figure 3.16, showing the highest activity for the VAP-ST2 sample to be at the 38th minute and the highest activity for the 2x FLAG tagged VAP to be at the 47th minute. There is a slight mismatch in the timing of the peak activity and the highest absorbance seen in Figure 3.16. However, that is due to the fact that the absorbance is measured slightly before the sample was eluted (making up to 1-2 minute difference).

Next a mixture of the two variants was loaded onto the column. After a couple of trial runs, a final experiment was carried out where each of the samples had the exact same amount of protein, namely 0.2 mg. One sample was of VAP-ST2, one of 2x FLAG tagged VAP and the third was a mixture of the two variants. All the samples contained 1 mM Na₂HPO₄ (to stabilize the monomers) and were stored for 5 days at RT to enable any swopping of the monomers should there be an equilibrium between the dimer and monomer form. All the three chromatographs acquired, one of the VAP-ST2, one of 2x FLAG tagged VAP and the mixture of the two variants can be seen in Figures A6 – A8 in appendix. The superimposition of the resulting chromatographs is shown in Figure 3.19.

![Figure 3.19 The separation of Strep-tagged VAP, 2x FLAG tagged VAP and a mixture of the two variants on a Q-Sepharose column. The chromatograms are superimposed on each other. Time is on the x-axis while the absorption at 280 nm is on the y-axis. All of the sample had 0.2 mg of protein, the mixture sample having 0.2 mg of each variant.](image)

A large peak was observed in all the samples between 60-65 min that was outside the concentration range of NaCl where the normal VAP elution occurs. The reason for this absorbance is presently unknown. Two peaks were seen in the mixture, but it was not possible to decide if a hybrid peak might be present in between them due to lack of resolution and overlap. The eluted peaks for the individual variants were different in height although the same amount was applied to the column. This indicated that the FLAG variant might be adsorbing to the equipment more than the StrepTag variant. However, the difference in height could also be partly due to the fact that the FLAG sample was centrifuged before it was loaded onto the column. In the mixture, the second peak was larger than the first which might suggest a hybrid. The elution flow of
these experiments was collected in small test tubes (1 ml/min) to be able to measure the activity and compare it to the elution peaks resulting from the Q-Sepharose column. The results are listed in Table A6. The highest activity measured in the mixture sample was in the samples eluted at the 52nd and 55th minute. Those results are in good accordance with the chromatogram in Figure 3.19 where the peak absorbance for the mixture sample is at the 50th and 53rd minute. The 2 minute difference results from the aforementioned fact that the absorbance is measured slightly before the samples are eluted.

The samples collected in the Q-Sepharose column separation of the mixture sample were activity measured and loaded onto SDS gels. The variants do travel identically in this electrophoresis system but information regarding the amount in each sample can be obtained. The result can be seen in Figure 3.20.

![Figure 3.20 SDS-PAGE electrophoresis of selected peaks from the Q-Sepharose column experiment performed with a mixture of VAP-ST2 and 2x FLAG tagged VAP. Well nr. 1 on the SDS gel corresponds to the sample eluted at the 49th minute in the column experiment and well 2 corresponds to the 50th minute of the column experiment and so on. A protein ladder (PageRuler™ Prestained Protein Ladder, Invitrogen) was loaded onto both ends of the gel, having molecular weight of the bands being (from top to bottom): 170, 130, 95, 72, 55, 43, 34, 26, 17, 11 kDa respectively.]

As can be seen in Figure 3.20, the protein samples migrated down to a location between the protein ladder bands of 55 and 72 kDa, which is in good accordance with the known value for the VAP enzyme. Compared to Figure 3.19, where the peaks of the mixture sample are shown to have the maximum absorbance at the 50th and 53rd minute, the greatest absorbance does seem to fit exactly the greatest protein concentration observed in Figure 3.20, since the greatest protein concentration is observed at the 52 – 56th
minute (wells 4-8), the 2-3 minute difference resulting from the aforementioned fact that the absorbance is measured slightly before the samples are eluted.

Finally, the samples from minute 48 – 58th were loaded onto a polyacrylamide gel for native proteins lacking SDS and electrophoresed to see if the variants would be clearly separable. From Figure 3.21 it can be seen that the two variants, VAP-ST2 and FLAG-tagged VAP, are almost undistinguishable regarding their migration down the gel. However, the FLAG-tagged VAP migrated a little bit further down the gel. No indications of a hybrid enzyme (two bands) was seen on the gel.

![Figure 3.21 PAGE electrophoresis of the flow-through samples from the Q-Sepharose column experiment performed with a mixture of VAP-ST2 and 2x FLAG tagged VAP. Well nr. 1 on the SDS gel corresponds to the sample eluted at the 48th minute in the column experiment and well 2 corresponds to the 49th minute of the column experiment and so on.](image)

3.8 Activity staining testing and analysis of the effects riboflavin has on VAP activity

Because no activity had been observed on the TUG-gels, it was decided to test the function of the main activity staining solution used, namely 2 mg of 5-bromo-4-chloro-3-indoyl phosphate disodium salt dissolved in 20 ml of activity staining buffer (1.0 M diethanolamine, 1.0 mM MgCl₂, pH 9.8) in a native gel made by the method of Laemmli. To do so, three types of AP (VAP, *E. coli* AP and calf intestinal AP) were electrophoresed on a polyacrylamide gel without added SDS and the gel then incubated into the activity staining solution. The results can be seen in Figure 3.22.
Figure 3.22 Testing of the function of the activity staining solution (2 mg of 5-bromo-4-chloro-3-indoyl phosphate disodium salt dissolved in 20 ml of activity staining buffer (1.0 M diethanolamine, 1.0 mM MgCl$_2$, pH 9.8)). The proteins were analysed by electrophoresis in a native version of the Laemmli buffer system (without SDS). In the well furthest on the left is the VAP-ST2 enzyme, in the well in the middle is an alkaline phosphatase derived from the bacterium E. coli and in the well to the left is an alkaline phosphatase derived from calf intestines.

As can be seen in Figure 3.22, the activity staining solutions worked very well with all three APs tested. That led to the conclusion that there might be something in the TUG-gels that was contributing to the inactivity of the enzymes. First to mind came riboflavin which is used to initiate the acrylamide polymerisation in the gels.

To find out the effect riboflavin has on the VAP enzyme, riboflavin (0.035 mg/ml) was added to a VAP-ST2 sample and the resulting solution was activity measured with the standard assay at 25°C using p-nitrophenyl phosphatase as substrate. Measurements were performed with a certain time interval as can be seen in Table 3.2.

Table 3.2 The effect of riboflavin on VAP activity. Activity measurements were performed with the standard assay at 25°C using p-nitrophenyl phosphatase as substrate and keeping the samples on ice. Triplicate measurements were performed with a certain time interval for a total of four times, the first one taking place after 10 minutes and the last one after a total of 24 hours. The results were averaged and are shown in the table along with the relative % of the activity compared to the initial control (bold). The control solution contained VAP-ST2 and TMC buffer while the other solution contained VAP-ST2 and 20 mM Tris, 10 mM MgCl$_2$, pH 8.0 buffer in addition to 0.035 mg/ml of riboflavin.

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Control [U/ml]</th>
<th>% control</th>
<th>Riboflavin added [U/ml]</th>
<th>% initial control</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>18.7</td>
<td>100</td>
<td>9.1</td>
<td>48.4</td>
</tr>
<tr>
<td>120</td>
<td>16.9</td>
<td>90</td>
<td>0.5</td>
<td>2.7</td>
</tr>
<tr>
<td>240</td>
<td>16.3</td>
<td>86.9</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>24 hr at RT (first 4h on ice)</td>
<td>8</td>
<td>42.8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
As can be seen from Table 3.2, the presumption proved to be true. Riboflavin was shown to diminish the activity of VAP very rapidly. After only 10 minutes the activity was only 48.4% compared to the initial control.

### 3.9 Experiments on TUG-gels lacking riboflavin

To find out if riboflavin is truly the cause of the inactivation of the enzyme in the gel, new gels were made which contained ammonium persulfate instead of riboflavin. Two VAP-ST2 samples having variable amount of protein (40 µg and 20 µg) were electrophoresed for 10 h at 4°C in the same buffer condition as before. After activity staining of those gels containing VAP-ST2 sample, a great deal of activity was observed as can be seen in Figure A10 and Figure A12 in the Appendix. Protein staining of these gels can also be seen in Figure A11 and Figure A13. As can be seen from these figures, the activity is only apparent in the 0 M urea solution half and according to the protein staining the folded and active enzyme migrates further down the gel than the inactive, unfolded enzyme, as was expected. Furthermore, Figure A11 indicates that both the dimer dissociation and the unfolding of the enzyme occurs simultaneously, i.e. that folded monomers do not form at any time during the unfolding process. Finally, a 2x FLAG tagged VAP sample, 20 µg, was electrophoresed at the exact same conditions as the VAP-ST2 samples were. The protein staining of the gel containing the 2x FLAG tagged VAP sample is shown in Figure A14. Unfortunately, the protein migrated through the gel in the time of the electrophoresis and, therefore, is not seen on the gel.
4 Discussions

The main aim of the project was to investigate the association of the VAP subunits by analysing the unfolding process taking place during transverse urea gradient gel electrophoresis. The second aim was to observe and identify possible subunit exchange of the VAP variants used and thus confirming the existence of a monomer-dimer equilibrium, by using ion exchange chromatography. In addition, the goal was to determine whether the association of the subunits was stronger or weaker between the two variants used (Strep-tagged and FLAG tagged VAP) and to determine whether the dimer association is partly dependent upon the magnesium ion in the active site. Furthermore, the aim was to analyse whether the results corresponded to the previously determined thermal stability and activity of the enzyme variants. From the results obtained, it is clear that further research is required to answer these questions. The main achievement of this project turned out to be the introduction and development of the transverse urea gradient gel electrophoresis method and to determine the best conditions for the analysis of VAP with the method. However, we had certain expectations in the beginning of the project about the possible outcome of the TUG-GE that were not fully realized.

The results obtained by Hjörleifsson and Ásgeirsson (2016) on the unfolding mechanism of VAP were used as a point of reference to determine the possible outcome and expectations of the TUG-GE of the VAP variants. As mentioned before, the conclusion was that the unfolding transition of VAP is most likely a three-step process taking place at 2 - 4 M urea concentration:

\[ N_2 \rightarrow I_2 \rightarrow 2I \rightarrow 2U \]

The intermediates forming were believed to be inactive dimers (I2), folded monomers (2I) and unfolded monomers (2U). Since the VAP monomer has a molecular mass of 56 kDa and the dimer is a larger molecule of 112 kDa, the monomer should travel a longer distance on a gel placed in an electric field than the dimer. As predicted, the monomers were shown to travel the longest distance. However, an unexpected observation was made regarding the travelling distance of the unfolded monomer, since it did not slow down under electrophoresis as expected if it had a larger Stoke's radius than the other forms. As expected, the unfolded monomers appeared as a larger size than the dimer (i.e. had shorter migration) in a size-exclusion chromatography experiment (Hjörleifsson & Asgeirsson, 2016). However, the expected TUG-GE results did not reflect that. Instead, an almost straight band was observed over the width of the gel with a slight curve downwards at the 2-4 M urea concentration region(possibly the 2I form). In some experiments the unfolded form (2U) would end at a slightly higher position on the gel compared to the folded form(s). However, if the inactive dimer (I2) is partially unfolded, the band would first have a slight bend upwards and then downwards, ending slightly higher on the gel than it started (2U). Overall, the difference in travel distance of the folded and unfolded forms was not great enough for VAP to make this method a reliable tool. However, some thoughts regarding the information that might be gained follow here.
In one of the first TUG-GE carried out on VAP-ST2 at RT, two downward curves were observed at the 0-4 M urea region (Figure 3.4). It is not fully understood what the curves are representing. However, since the upward incline of the band indicates the subsequent unfolding of the protein as the urea concentration increases in the gel, the curve region is most likely representing the dissociation event of the dimer. Furthermore, the protein band showed shorter travelling distance at the 8 M urea part of the gel than in the 0 M urea part, i.e. the folded protein had greater migration than the unfolded protein, which was to be expected.

The results attained from the TUG-GE of native VAP-ST2 at 4°C differed considerably between different experiments, possibly in some cases due to the amount of protein applied to the gels. At first, the typical results had a single protein band forming a large upward curve at the 0-4 M urea region (Figure 3.5 and Figure 3.6). This curve could possibly indicate the formation of a slightly unfolded dimer intermediate (I2) which would migrate as a slightly larger species. The subsequent dissociation of the dimer intermediate to folded monomers would result in a greater migration that the downward ending of the curve could be indicating. Finally, a slight upward incline was noticed in the otherwise straight protein band from the ending of the curve to the end of the gel (at the 8 M urea region). This incline could possibly be indicating the unfolding of the monomers. However, the protein band did begin and end at a similar height in the gel, i.e. indicating that the folded enzyme had similar migration as the unfolded monomers which was not to be expected.

Figure 3.7 shows the results of a TUG-GE of VAP-ST2 performed in the exact same conditions as before, except for having a bit lower current during the electrophoresis, namely 30 mA instead of 37 mA (for two gels). Three protein bands were observed. An assumption was made, after comparing all the TUG-GE results, that having a lower current during the electrophoresis would generally result in more protein bands, i.e. greater separation of species. The amount of protein did also seem to affect the number of protein bands observed, as can be seen by a comparison of Figure 3.14 to Figure 3.15 where the only difference was the amount of protein applied to the gel. Loading greater amount of protein onto the gels generally resulted in two or more protein bands whereas loading less amount most often resulted in only one protein band. However, some exceptions were noticed. One possible reason for the variety of the number of protein bands is deamination and the consequent formation of a molecular species having different net charge compared to the initial enzyme.

The results of only one of the two TUG-GE performed of denatured VAP-ST2 samples (treated with heat and high urea concentrations before electrophoresis) was of good enough gel-quality to be preserved and photographed (Figure 3.8). Two relatively straight protein bands were observed. It is thought that the more intensive band is representing the unfolded enzyme while the other weaker band is some sort of aggregated protein. Furthermore, because of the heating of the urea denaturated samples, there is a possibility of cyanate formation and consequently, also a possibility of carbamylation of the proteins. No indication of refolding was observed.

The addition of DTT to the TUG-gels did not seem to affect the results and, therefore, did not confirm the theory that the aggregated protein was a result of sulphide bond formation. This was concluded based on the results seen in Figure 3.10, where three protein bands were observed.
A couple of electrophoresis runs were performed on dual 0/8 M urea gels (sharp boundary with no intentional gradient), in the hope of clearer results. As before, the electrophoresis of denatured VAP-ST2 resulted in a relatively straight protein band throughout the gel, only having some minor curves at the 8 M urea end. Those results confirm the fact that the enzyme does not manage to refold during the time of the electrophoresis.

Four electrophoresis experiments on dual 0/8 M dual urea gels were performed on native VAP-ST2 samples, all of which resulted in somewhat different results, although the condition for the electrophoresis was the same for all of them. The first one (14 µg protein) resulted in 3 bands, the second and the third ones (14 µg and 12 µg respectively) in one protein band that faded out in the 8 M urea part of the gel and the fourth and final one (40 µg) in two protein bands. As mentioned in the results section, the fading of the bands in the second and third electrophoresis, was possibly due to a slight incline of the sample well of the gels. Hence, in future experiments, an attention should be paid to the incline of the sample wells and efforts should be made to eliminate the problem of uneven sample distribution.

Overall, the results attained from the electrophoreses of VAP were not always consistent. For example, a distinct difference in the migration between the folded enzyme compared to the unfolded monomers was not always observed, although it was to be expected based on the conclusions made by Hjörleifsson and Ásgeirsson (2016) that the unfolded monomers would migrate a shorter distance. The number of protein bands was also inconsistent throughout the experiments. Possible reasons being as mentioned before, the various amount of protein used and the alterations in the current used during the electrophoreses. The incompatibility in the results could also, to some extent, result from some artefacts of the electrophoreses. Furthermore, there is always the possibility that the minor differences in the treatment of the sample used, e.g. the time they were exposed at RT, could be causing the various results observed.

An important discovery was made regarding riboflavin, the photocatalyst used in the gels. Riboflavin was proved to cause the inactivation of the VAP enzyme, which explained why no activity was measured after the TUG-GE experiments with in-gel activity stains. Following the discovery, a couple of gels were made lacking riboflavin. Indeed, activity staining of VAP worked on those gels. Furthermore, one of the gels (Figure A11) showed indication that the dimer dissociation and the unfolding of the enzyme could be occurring simultaneously, i.e. that folded monomers did not form at any time during the unfolding process.

The six additional negative charges that come with the addition of the FLAG-tag to the VAP enzyme are possibly making the variant more convenient for TUG-GE than the Strep-tagged variant. This was concluded based on the few experiments performed on FLAG-tagged VAP. For example, when both the variants were electrophoresed on TUG-gels in the exact same condition, the results seemed to be much clearer for the FLAG-tagged variant. Three protein bands were observed for the FLAG-tagged variant whereas only one was observed for the Strep-tagged one (Figure 3.9 and Figure 3.10).

Due to time constraints, not enough electrophoreses runs were carried out to produce consistent enough results to make strong assertions about the unfolding process of VAP and the association of its monomers. Future experimentation will include more
electrophoreses of both Strep-tagged and FLAG-tagged VAP on dual gels lacking riboflavin with the aim of getting more consistent results. The dual gels seem to be ideal for continuous studies, at least during further establishment of the method, since the dual gels result in a simpler outcomes that facilitates the reading of the patterns produced.

In the attempt to see if there exists a monomer-dimer equilibrium in VAP, a mixture of the two VAP variants (Strep-tagged and FLAG-tagged VAP) was applied to a Q-Sepharose column. The expectation was to see three peaks, one for each variant and a third one located between them, representing the hybrid enzyme. Before the experiments with the mixture were carried out, it was verified that the variants were indeed separable on the column. They proved to be so, eluting at gradient values differing in about 10 mS/cm.

The results of the separation of the variant mixture on a Q-Sepharose column was two merged peaks, one being slightly broader than the other (Figure 3.19). Since only two merged peaks appeared, the hypothesis was not confirmed. However, the results did not exclude the possibility of a hybrid enzyme formation and, thus, the existence of a dynamic equilibrium between the monomeric and dimeric form.

The results acquired on the separation of the variants are maybe not so surprising considering a recent study on the equilibrium constant between the dimer and the monomers of VAP which states that the equilibrium constant is $K_d \approx 10^{-100}$ nMol at pH 8.0 for the dimer monomer equilibrium (Unpublished observations of Jens G. Hjörleifsson). That means that the chances of monomer sipping are very low since the dimers rarely disassociate. At any given time, only about $1:10^9$ of the protein is in the monomeric form. Furthermore, the monomeric form was also shown to be very short lived, since the on-rate is very fast and the off-rate is very slow. Therefore, the likelihood for a situation where Strep-tagged and FLAG-tagged monomers are replaced in such a way that allows for monomer sipping, is very low. Given this information, the analysis of the hybrid enzyme formation is very hard using a Q-Sepharose column, maybe even impossible. However further studies should be performed to fully determine this, possibly using double-labelled variants (i.e one with green and the other with red fluorescent probes).
References


Papaleo, E., Renzetti, G., Invernizzi, G., & Asgeirsson, B. (2013). Dynamics fingerprint and inherent asymmetric flexibility of a cold-adapted homodimeric enzyme. A

ProtParam. Retrieved from http://web.expasy.org/cgi-bin/protparam/protparam1?Q93P54@noft@


Appendix

Figures

*Figure A1* The initial setup where an electrophoresis chamber from Invitrogen was used.
Figure A2 The second setup where an electrophoresis chamber from Hoefer was used.
Figure A3 The experimental setup for the preparation of the transverse urea gradient gels. In front of the peristaltic pump there is a mixing chamber that sits on top of a magnetic stirrer. A reservoir of 8 M urea gel solution is on the right side of the pump and the casting box is on the left. The casting box is clear and plastic and in it are the three gel sandwiches. On the bottom left on the casting box is a inlet for the gel solution. Gel solutions containing 0 M urea is placed in the mixing chamber whilst the 8 M urea solution is placed in the chamber on the right. As the solution from the mixing chamber is pumped into the casting box, using two channels of the pump, the 8 M urea solution in the chamber on the right is pumped in using only one channel of the pump. The outcome is a linear gradient with an increasing urea concentration in the mixing chamber.
Figure A4 TUG-GE of VAP-ST2 performed on a gel containing 10 mM DTT, at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) electrophoresis buffer. The sample contained 28 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The sample was electrophoresed for 5 hours. The current was first set to 80 mA, however it was soon lowered to 40 mA.
Figure A5 TUG-GE of bacterial α-amylase from Bacillus subtilis performed on a gel containing 10 mM DTT, at 4°C in 0.5 M Tris/0.5 M Bicine (pH 8.4) electrophoresis buffer. The sample contained 50 µg protein and the ratio of protein versus sample buffer was 1:1 (v/v). The sample was electrophoresed for 5 hours and the current was set to 15 mA.
Figure A6 The chromatograph of the Strep-tagged VAP sample loaded on a Q-Sepharose column and used for the comparison with FLAG-tagged VAP and a mixture sample. Time is on the x-axis while both absorption at 280 nm and the salt gradient are on the y-axis (on either site). The Strep-Tag VAP sample contained 0.2 mg of protein.

Figure A7 The chromatograph of the FLAG-tagged VAP sample loaded on a Q-Sepharose column and used for the comparison with Strep-tagged VAP and a mixture sample. Time is on the x-axis while both absorption at 280 nm and the salt gradient are on the y-axis (on either site). The FLAG-Tag VAP sample contained 0.2 mg of protein.
Figure A8 The chromatograph of the mixture VAP sample loaded on a Q-Sepharose column and used for the comparison with a Strep-tagged VAP and FLAG-tagged VAP samples. Time is on the x-axis while both absorption at 280 nm and the salt gradient are on the y-axis (on either site). The mixture sample contained 0.2 mg of ST2-VAP and 0.2 mg of FLAG-tagged VAP.
Figure A9 SDS-PAGE results of the purification of alkaline phosphatase from the cold adapted bacteria Vibrio sp. G15-21 (VAP). The protein ladder is in the leftmost well (PageRuler™ Prestained Protein Ladder, Invitrogen), the molecular weight of the bands are (from top to bottom): 170, 130, 95, 72, 55, 43, 34, 26, 17, 11 kDa respectively. The protein samples that are relevant are in wells 2-5 following the ladder. The other samples are not relevant. Well 2 contains the flow through sample, well 3 contains the culture sample, well 4 contains sample 1 (elute I) and well 5 contains sample 2 (elute II). Each sample contained 30 µl protein and 10 µl LDS sample buffer. As can be seen the protein purification process succeeded, only one dominant band appeared in both wells 4 and 5 that fits to the size of the VAP enzyme (between the protein ladder bands of 55 – 72 kDa).
Figure A10 Activity staining of VAP-ST2 electrophoresed on a dual gel having 0 M urea solution in one half (left) and 8 M urea solution in the other half (right) and containing sodium persulfate instead of riboflavin. The amount of protein was 20 µg, the electrophoresis lasted 10 h (10 mA) and the gel had been submerged in activity staining solution for 15 minutes and then transferred to water-30% (v/v) methanol.

Figure A11 Protein staining with Silver Blue of VAP-ST2 electrophoresed on a dual gel having 0 M urea solution in one half (left) and 8 M urea solution in the other half (right) and containing sodium persulfate instead of riboflavin. The amount of protein was 20 µg and the electrophoresis lasted 10 h (10 mA).
**Figure A12** Activity staining of VAP-ST2 electrophoresed on a dual gel having 0 M urea solution in one half (left) and 8 M urea solution in the other half (right) and containing sodium persulfate instead of riboflavin. The amount of protein is 56 µg, the electrophoresis lasted 10 h (10 mA) and the gel had been submerged in activity staining solution for 15 minutes and then transferred to water-30% (v/v) methanol.

**Figure A13** Protein staining with Coomassie Blue of VAP-ST2 electrophoresed on a dual gel having 0 M urea solution in one half and 8 M urea solution in the other half and containing sodium persulfate instead of riboflavin. The left half of the gel contains 0 M urea solution while the right half contains 8 M urea solution. The amount of protein was 56 µg and the electrophoresis lasted 10 h (10 mA).
Figure A14 Protein staining with Coomassie Blue of 2x FLAG tagged VAP electrophoresed on a dual gel having 0 M urea solution in one half and 8 M urea solution in the other half and containing sodium persulfate instead of riboflavin. The left half of the gel contains 0 M urea solution while the right half contains 8 M urea solution. The amount of protein is 20 µg and the electrophoresis lasted 10 h (10 mA). Unfortunately, the protein migrated through-out the gel in the time of the electrophoresis and therefore is not seen.
### Tables

**Table A1** The recipes for the Urea/acrylamide gel solutions containing a photopolymerization catalyst (riboflavin).

<table>
<thead>
<tr>
<th>Solutions</th>
<th>The 0 M urea/15% acrylamide gel solution</th>
<th>The 8 M urea/11% acrylamide gel solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid urea (g)</td>
<td>-</td>
<td>14.4</td>
</tr>
<tr>
<td>Acrylamide/bis acrylamide stock solution (mL) [30:08]</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>10x Electrophoresis buffer (mL)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0.04 mg/ml riboflavin (mL)</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O (mL)</td>
<td>8.25</td>
<td>1.2</td>
</tr>
<tr>
<td>Optional: DTT (mM)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>TEMED (µL)</td>
<td>36</td>
<td>36</td>
</tr>
</tbody>
</table>

**Table A2** The recipe for the Urea/acrylamide gel solutions containing ammonium persulfate instead of riboflavin.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>The 0 M urea/15% acrylamide gel solution</th>
<th>The 8 M urea/11% acrylamide gel solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid urea (g)</td>
<td>-</td>
<td>14.4</td>
</tr>
<tr>
<td>Acrylamide/bis acrylamide stock solution (mL) [30:08]</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>10x Electrophoresis buffer (mL)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ammonium persulfate 10% (w/v)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O (mL)</td>
<td>8.25</td>
<td>1.2</td>
</tr>
<tr>
<td>Optional: DTT (mM)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>TEMED (µL)</td>
<td>36</td>
<td>36</td>
</tr>
</tbody>
</table>
Table A3 The recipe for the basic protein sample buffer.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>(% (w/v))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>50</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>0.2</td>
</tr>
<tr>
<td>H₂O</td>
<td>49.8</td>
</tr>
</tbody>
</table>

Table A4 The composition for the electrophoresis buffers used.

<table>
<thead>
<tr>
<th>0.5 M Tris-acetate, pH 8.0 (10x)</th>
<th>0.5 M Tris/0.5 M Bicine, pH ~ 8.4 (10x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris</td>
<td>0.5 M Tris</td>
</tr>
<tr>
<td>Acetic acid to pH 8.0</td>
<td>0.5 M Bicine</td>
</tr>
</tbody>
</table>

Table A5 Enzyme activity measurements of samples (seen in Figure 3.16) loaded on a Q-Sepharose column. On the left are the results from the VAP-ST2 sample and to the right are the results from the 2x FLAG tagged VAP sample. The activity measurements were performed with the standard assay at 25°C using p-nitrophenyl phosphatase as substrate. The samples were diluted 100-fold and the dilution was accounted for in the activity calculations.

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>ΔA</th>
<th>Activity [U/mL]</th>
<th>Time [min]</th>
<th>ΔA</th>
<th>Activity [U/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>0.036</td>
<td>3.6</td>
<td>42</td>
<td>0.013</td>
<td>1.3</td>
</tr>
<tr>
<td>37</td>
<td>0.156</td>
<td>15.6</td>
<td>43</td>
<td>0.017</td>
<td>1.7</td>
</tr>
<tr>
<td>38</td>
<td>0.467</td>
<td>46.7</td>
<td>44</td>
<td>0.128</td>
<td>12.8</td>
</tr>
<tr>
<td>39</td>
<td>0.12</td>
<td>12</td>
<td>45</td>
<td>0.355</td>
<td>35.5</td>
</tr>
<tr>
<td>40</td>
<td>0.274</td>
<td>27.4</td>
<td>46</td>
<td>0.578</td>
<td>57.8</td>
</tr>
<tr>
<td>41</td>
<td>0.222</td>
<td>22.2</td>
<td>47</td>
<td>0.905</td>
<td>90.5</td>
</tr>
<tr>
<td>42</td>
<td>0.038</td>
<td>3.8</td>
<td>48</td>
<td>0.46</td>
<td>46</td>
</tr>
<tr>
<td>43</td>
<td>-</td>
<td>-</td>
<td>49</td>
<td>0.414</td>
<td>41.4</td>
</tr>
<tr>
<td>44</td>
<td>0.017</td>
<td>1.7</td>
<td>50</td>
<td>0.25</td>
<td>25</td>
</tr>
<tr>
<td>45</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>0.006</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.006</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A6 Enzyme activity measurements of samples seen in Figure 3.17 loaded on a Q-Sepharose column. On the left are the results from the VAP-ST2 sample, in the middle are the results from the 2x FLAG tagged VAP sample and to the right are the results from the VAP-ST2-FLAG-tag mixture. The activity measurements were performed with the standard assay at 25°C using p-nitrophenyl phosphatase as substrate. The samples were diluted 100-fold and the dilution was accounted for in the activity calculations.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>-</td>
<td>-</td>
<td>49</td>
<td>0.004</td>
<td>2.2</td>
<td>43</td>
<td>0.001</td>
<td>0.8</td>
</tr>
<tr>
<td>52</td>
<td>0.341</td>
<td>182.5</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>44</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>53</td>
<td>-</td>
<td>-</td>
<td>51</td>
<td>0.066</td>
<td>35.3</td>
<td>45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>54</td>
<td>0.916</td>
<td>489.4</td>
<td>52</td>
<td>-</td>
<td>-</td>
<td>46</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>-</td>
<td>-</td>
<td>53</td>
<td>0.112</td>
<td>60</td>
<td>47</td>
<td>0.001</td>
<td>0.7</td>
</tr>
<tr>
<td>56</td>
<td>0.166</td>
<td>99.7</td>
<td>54</td>
<td>-</td>
<td>-</td>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>57</td>
<td>-</td>
<td>-</td>
<td>55</td>
<td>0.062</td>
<td>33.3</td>
<td>49</td>
<td>0.03</td>
<td>16.1</td>
</tr>
<tr>
<td>58</td>
<td>0.063</td>
<td>34</td>
<td>56</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>59</td>
<td>-</td>
<td>-</td>
<td>57</td>
<td>0.031</td>
<td>17</td>
<td>51</td>
<td>0.129</td>
<td>69.4</td>
</tr>
<tr>
<td>60</td>
<td>0.003</td>
<td>1.8</td>
<td>58</td>
<td>-</td>
<td>-</td>
<td>52</td>
<td>1.142</td>
<td>610.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>59</td>
<td>0.034</td>
<td>18.2</td>
<td>53</td>
<td>0.747</td>
<td>399.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>54</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61</td>
<td>0.027</td>
<td>14.9</td>
<td>55</td>
<td>1.009</td>
<td>539.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>62</td>
<td>-</td>
<td>-</td>
<td>56</td>
<td>0.283</td>
<td>151.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63</td>
<td>0.035</td>
<td>18.8</td>
<td>57</td>
<td>0.103</td>
<td>55.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65</td>
<td>0.03</td>
<td>16.3</td>
<td>59</td>
<td>0.054</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66</td>
<td>-</td>
<td>-</td>
<td>60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>67</td>
<td>0.022</td>
<td>11.9</td>
<td>61</td>
<td>0.03</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>62</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>69</td>
<td>-</td>
<td>-</td>
<td>63</td>
<td>0.022</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>70</td>
<td>-</td>
<td>-</td>
<td>64</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>71</td>
<td>0.012</td>
<td>6.7</td>
<td>65</td>
<td>0.005</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>66</td>
<td>0.019</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>73</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>74</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>75</td>
<td>0.009</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>