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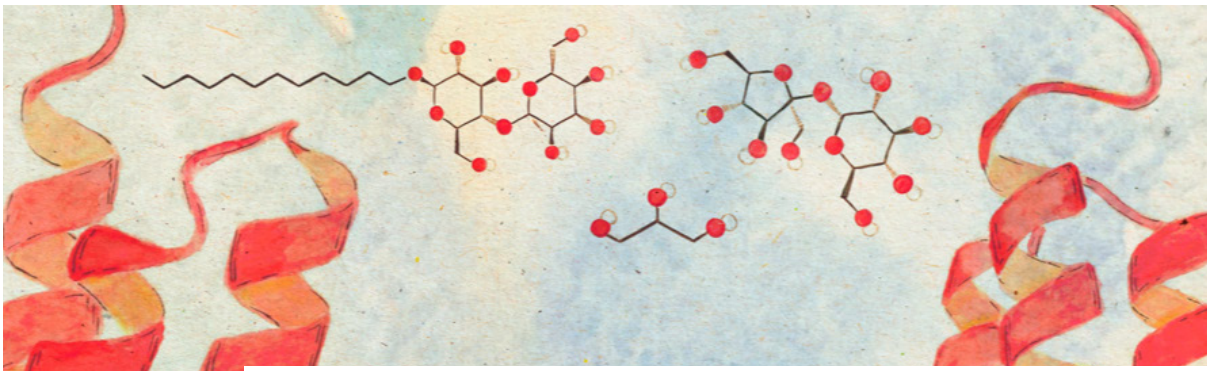

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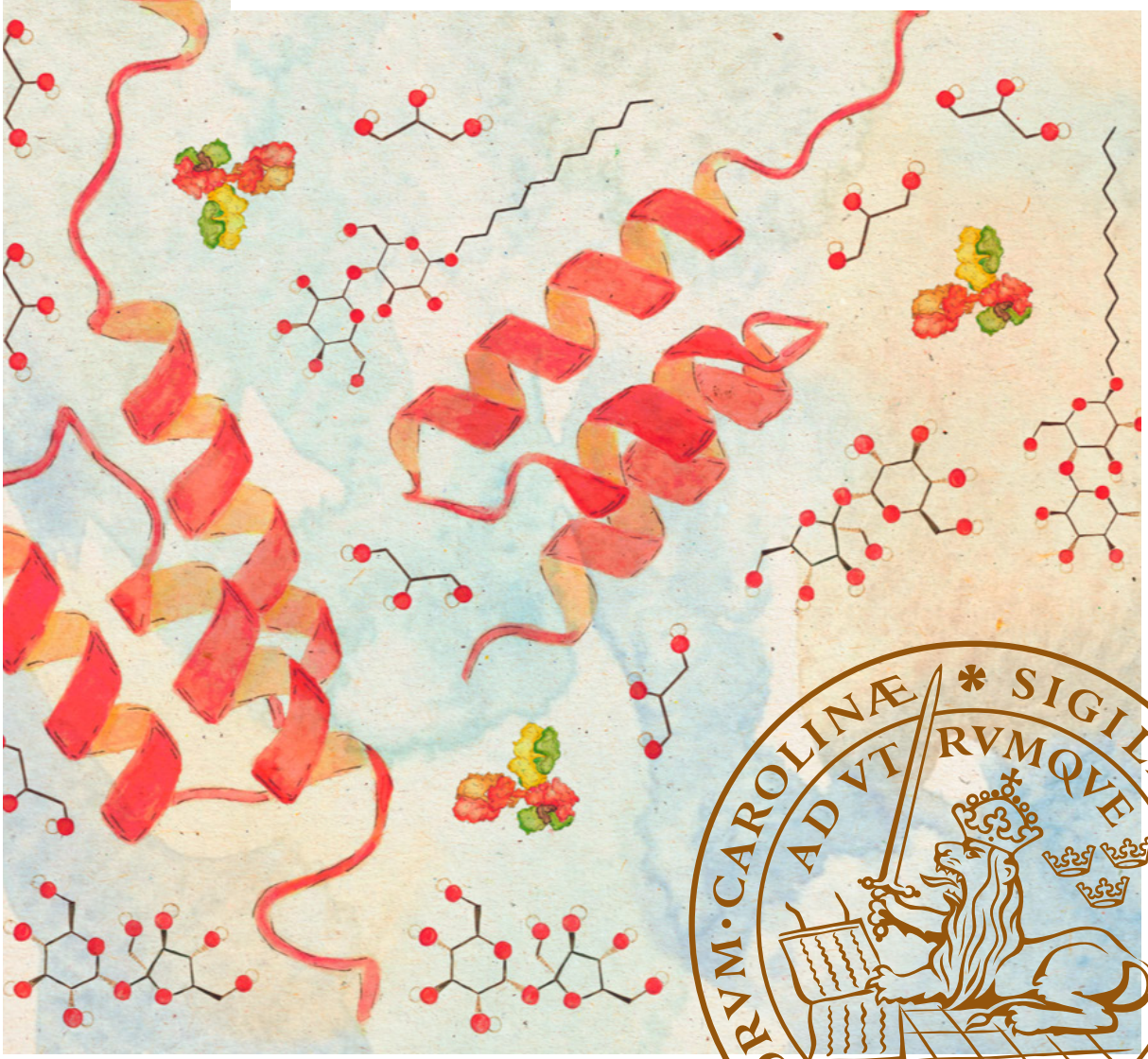

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Ingrid Ramm

LUND UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on 14th of March at 09.00 in hall KC:C, Department of Process and Life Science Engineering, Division of Food and Pharma, Naturvetarvägen 22 Lund

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Title and subtitle: Stability of therapeutic proteins in solution:
The impact of excipients on chemical and physical degradation

Abstract: Therapeutic proteins replicate the functions of naturally occurring proteins in the body, offering treatments for various diseases, including cancer. However, their efficacy, safety, and function can be compromised by chemical and physical degradation, such as deamidation and aggregation, which can occur during production, transportation, storage, or after administration. Therefore, stabilizing formulations are essential to maintain their native structure and prevent degradation. This thesis investigates how excipients can enhance the stability of therapeutic proteins in liquid formulations, with a focus on chemical stability. It also aims to develop an in vitro methodology for predicting the stability and behavior of therapeutic proteins in vivo.

The study examines the effect of six excipients; glycerol, fructose, sucrose, melezitose, polysorbate 80, and n-Dodecyl-β-D-maltoside, on the chemical stability of the therapeutic affibody GA-Z. An accelerated stability study is used to assess the impact of these excipients. The results show that glycerol, fructose, sucrose, and melezitose enhance the chemical stability of GA-Z by reducing deamidation, isomerization, and hydrolysis. These excipients increase the chemical stability by stabilizing the folded state of the z-domain and inducing a small structural change, which lowers the conformational flexibility of the peptide backbone of GA-Z. In contrast, polysorbate 80 does not affect the stability or structure of GA-Z, while n-Dodecyl-β-D-maltoside induces significant changes in both stability and structure, possibly through micelle-protein complex formation. Additionally, a novel in vitro methodology combining Asymmetrical Flow Field-Flow Fractionation and Liquid Chromatography-Mass Spectrometry was developed, capable of predicting in vivo stability, analyzing serum albumin binding, detecting changes in protein size, and identifying aggregates.

These findings provide valuable insights into the stabilization of therapeutic proteins in solution and present a useful method for predicting their stability and behavior in vivo. The results provided by this study will aid in the development of more effective protein formulations by enhancing stability, minimizing degradation, and advancing the development of safer and more efficient protein drugs.

Keywords: Therapeutic protein, protein structure, polyol, glycerol, sucrose, surfactant, n-Dodecyl-β-D-maltoside, polysorbate 80, deamidation, isomerization, stability, liquid formulation

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”

- Marie Curie

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Abstract

Therapeutic proteins replicate the functions of naturally occurring proteins in the body, offering treatments for various diseases, including cancer. However, their efficacy, safety, and function can be compromised by chemical and physical degradation, such as deamidation and aggregation, which can occur during production, transportation, storage, or after administration. Therefore, stabilizing formulations are essential to maintain their native structure and prevent degradation. This thesis investigates how excipients can enhance the stability of therapeutic proteins in liquid formulations, with a focus on chemical stability. It also aims to develop an *in vitro* methodology for predicting the stability and behavior of therapeutic proteins in vivo.

The study examines the effect of six excipients; glycerol, fructose, sucrose, melezitose, polysorbate 80, and n-Dodecyl-β-D-maltoside, on the chemical stability of the therapeutic affibody GA-Z. An accelerated stability study is used to assess the impact of these excipients. The results show that glycerol, fructose, sucrose, and melezitose enhance the chemical stability of GA-Z by reducing deamidation, isomerization, and hydrolysis. These excipients increase the chemical stability by stabilizing the folded state of the z-domain and inducing a small structural change, which lowers the conformational flexibility of the peptide backbone of GA-Z. In contrast, polysorbate 80 does not affect the stability or structure of GA-Z, while n-Dodecyl-β-D-maltoside induces significant changes in both stability and structure, possibly through micelle-protein complex formation. Additionally, a novel *in vitro* methodology combining Asymmetrical Flow Field-Flow Fractionation and Liquid Chromatography-Mass Spectrometry was developed, capable of predicting *in vivo* stability, analyzing serum albumin binding, detecting changes in protein size, and identifying aggregates.

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Populärvetenskaplig sammanfattning

Proteiner har många viktiga funktioner i våra kroppar och inom medicin används terapeutiska proteiner för att behandla flera olika typer av sjukdomar. Terapeutiska proteiner är dock ömtåliga och kan brytas ner, vilket kan påverka deras funktion och effektivitet. På grund av proteinernas känslighet försöker forskare att hitta nya verktyg för att göra dem mer stabila och förhindra att de bryts ner.

Terapeutiska proteiner kan brytas ner genom flera olika processer. Nedbrytningen kan försämra proteinernas förmåga att fylla sin funktion som läkemedel. Nedbrytningen kan också leda till en oönskad aktivering av immunförsvaret, vilket i sin tur kan ge upphov till farliga biverkningar. En annan stor utmaning är att de ofta behöver förvaras vid mycket låga temperaturer eller torkas till pulver för att förhindra att de bryts ner. Även om dessa metoder kan skydda proteinerna, gör det också att distribution och användning blir komplicerad och dyr. Det är viktigt att förbättra de terapeutiska proteinernas stabilitet eftersom det kan minska behovet av dessa dyra och omständliga stabiliseringsmetoder och bidra till att göra terapeutiska proteinläkemedel mer tillgängliga för patienter runt om i världen.

GA-Z tillhör proteingruppen Affibody som är en allt mer vanlig förekommande terapeutisk proteingrupp. GA-Z bryts lätt ner genom två kemiska reaktioner som kallas deamidering och isomerisering och har därför valts som modell-protein i denna studie. Vi har undersökt om glycerol, socker och detergenter kan hjälpa till att skydda GA-Z från att brytas ner. Glycerol är en liten organisk molekyl och detergenter används ofta i tvättmedel. Våra undersökningar visade att glycerol och sockerarterna, fruktos, sackaros och melezitos förhindrar deamidering och isomerisering. Undersökningar visade också att glycerol och socker gör att proteinet GA-Z blir mer stabilt genom att skydda proteinets struktur och genom att göra strukturen stelare. Vi testade vidare vad olika detergenter hade för inverkan på GA-Z. Den testade detergenten, polysorbat 80, hade ingen effekt på det terapeutiska proteinets stabilitet, medan en annan detergent, n-Dodecyl-β-D-maltosid, både minskade och ökade nedbrytningen. Resultaten visar att glycerol och socker kan användas för att göra proteinläkemedel mer stabila och att detergenter bör användas med försiktighet i proteinläkemedel, eftersom de ibland kan påverka stabiliteten negativt. Vi fann också att vissa egenskaper hos GA-Z struktur gör den mer benägen

att brytas ner. Att förstå vilka strukturegenskaper som gör proteiner känsliga för nedbrytning kan göra det lättare för forskare att designa mer stabila och effektiva proteinläkemedel i framtiden.

Själva testet av förmågan hos glycerol, socker och detergenter att stabilisera GA-Z genomfördes i flera steg. Först lagrades GA-Z med respektive och utan dessa ämnen vid 37 °C i upp till 44 dagar. Därefter uppmättes hur mycket GA-Z som bröts ner i respektive fall. Nedbrytningen mättes genom att använda tekniken Liquid Chromatography-Mass Spectrometry. För att förstå varför glycerol, socker och detergenterna påverkar nedbrytningen undersöktes proteinets struktur med flera avancerade tekniker, som AlphaFold2, Nuclear Magnetic Resonance spectroscopy och Small Angle Neutron Scattering.

Ett terapeutiskt protein som har injicerats i kroppen kan bryta ner eller interagera med andra komponenter i blodomloppet på flera olika sätt. Vi har därför utvecklat en ny metod för att studera proteiners egenskaper och stabilitet i plasma för att bättre förstå hur proteiner beter sig i blodomloppet. Denna metod kan användas innan stabilitetstudier görs på djur eller patienter. Den utvecklade metoden är en kombination av teknikerna Asymmetrical Flow Field-Flow Fractionation and Liquid Chromatography-Mass Spectrometry och kommer att kunna hjälpa forskare att identifiera hur proteiner beter sig i kroppen. Vid utvecklingen av nya läkemedel kan detta spara tid och pengar eftersom det gör det möjligt för forskare att testa proteiners beteende i ett tidigare skede av processen än vad som är möjligt idag.

Sammantaget ger denna studie värdefulla kunskaper om hur terapeutiska proteiner kan stabiliseras. Den bidar också med en ny metod som kan användas för att testa proteiners stabilitet och egenskaper i plasma, vilket kan påskynda utvecklingen av nya proteinläkemedel.

Popular scientific summary

Proteins are essential molecules in our bodies, responsible for a wide range of functions. In medicine, therapeutic proteins are used to treat many diseases. However, therapeutic proteins are fragile and can break down or change in ways that affect their function and effectiveness. That is why researchers are trying to find ways to keep these therapeutic proteins stable.

Therapeutic proteins break down through various processes, e.g. aggregation, deamidation, and isomerization. These changes can reduce the ability of therapeutic proteins to do their job and even trigger unwanted immune responses, leading to dangerous side effects. Often, proteins need to be kept at very low temperatures or be dried into powders to prevent degradation processes. While these methods help protect the proteins, they also make distribution and use more complicated and expensive. Improving protein stability could reduce the need for these costly and inconvenient stabilization methods and help make protein treatments more accessible to patients around the world.

In this research, we studied how the molecules glycerol, sugars, and detergents can help protect the therapeutic protein GA-Z from breaking down. GA-Z is known to degrade easily through two chemical reactions called deamidation and isomerization. The results show that glycerol and the sugars fructose, sucrose, and melezitose can prevent deamidation and isomerization. They stabilize GA-Z by protecting the structure of GA-Z and by making the structure stiffer. On the other hand, the detergent, polysorbate 80, does not affect the stability of GA-Z, while another detergent, n-Dodecyl-β-D-maltoside, both reduces and increases degradation. The results show that glycerol and sugars can be used to keep therapeutic proteins stable and prevent breakdown reactions, while the detergents need to be used with caution in protein-based drugs, as they can sometimes affect stability negatively. We also found that some characteristics of the structure of GA-Z itself make it prone to degradation. Understanding which parts of a protein are most vulnerable to degradation can help scientists design better and more stable drugs in the future.

To test the stabilizing effects of glycerol, sugars, and detergents, we stored GA-Z at 37 °C for up to 44 days and measured how much it degraded in the presence and absence of the molecules. We used a technique called Liquid Chromatography-Mass Spectrometry to measure the degradation. To better understand how glycerol,

sugars, and detergents affect the structure of the protein, we combined several other advanced techniques, like AlphaFold2, Nuclear Magnetic Resonance spectroscopy, and Small Angle Neutron Scattering.

When a therapeutic protein is injected into the body, it can break down or interact with blood components. To better understand how proteins behave in the bloodstream, we developed a new method to study their stability in plasma before testing them in animals or patients. This method combines Asymmetrical Flow Field-Flow Fractionation and Liquid Chromatography-Mass Spectrometry and will help researchers identify how proteins behave in the body. This could save time and money in the development of new drugs by allowing scientists to test protein behavior earlier in the development process.

This research provides valuable insights into how therapeutic proteins can be stabilized, and help improve their effectiveness and safety. It also offers new tools for testing protein behavior, potentially speeding up the development of new protein drugs and save resources along the way.

List of papers

Paper I

Ingrid Ramm, Adrian Sanchez-Fernandez, Jaeyeong Choi, Christian Lang, Jonas Fransson, Herje Schagerlöf, Marie Wahlgren, Lars Nilsson. The impact of glycerol on an affibody conformation and its correlation to chemical degradation. Pharmaceutics. 2021;12:1853

Paper II

Ingrid Ramm, Carl Diehl, Amanda Västberg, Natalia Markova, Herje Schagerlöf, Marie Wahlgren, Lars Nilsson. The effect of glycerol and protein structure on chemical degradation through deamidation and isomerization.

Paper III

Ingrid Ramm, Carl Diehl, Amanda Västberg, Johanna Hjalte, Herje Schagerlöf, Marie Wahlgren, Lars Nilsson. The effect of pharmaceutical excipients on chemical degradation through deamidation and isomerization.

Paper IV

Ingrid Ramm, Mats Leeman, Herje Schagerlöf, Ileana Rodríguez León, Alejandra Castro, Lars Nilsson. Investigation of native and aggregated therapeutic proteins in human plasma with asymmetrical flow field-flow fractionation and mass spectrometry. Analytical and Bioanalytical Chemistry. 2022;414:8191-8200

Contributions to papers

Paper I

I designed the stability study together with Jaeyeong Choi, Jonas Fransson, and my supervisors, Lars Nilsson and Marie Wahlgren. I designed the combination of techniques used to characterize glycerol-induced stabilization and structural changes with Lars Nilsson and Marie Wahlgren. I developed the LC and LC-MS method with Herje Schagerlöf and the AF4 method with Lars Nilsson. I performed the stability study, the water activity measurements, the LC analysis, the LC-MS experiments, and the Asymmetrical Flow Field-Flow Fractionation experiments. I also performed the SANS experiments together with Adrian Sanchez-Fernandez and Christian Lang. I analyzed and processed the LC and LC-MS data with Herje Schagerlöf, the AF4 data with Lars Nilsson, and the SANS data with Adrian Sanchez-Fernandez. I wrote the original draft together with Adrian Sanchez-Fernandez and finalized the paper together with all the authors.

Paper II

I designed the stability study together with my supervisors, Lars Nilsson and Marie Wahlgren. I designed the combination of techniques used to characterize glycerol-induced stabilization and structural changes with Carl Diehl, Lars Nilsson, and Marie Wahlgren. I developed the trypsin digestion method and the LC-MS method with Herje Schagerlöf. I performed the stability study, the trypsin digestion, the LC-MS experiments, and the DSC experiments with Amanda Västberg. I also prepared samples for the SUPR-DSF and NMR experiments. I analyzed and processed the LC-MS and NMR data with Carl Diehl. I performed the structural analysis on GA-Z with an AlphaFold2-generated structure. I wrote the original draft together with Carl Diehl and finalized the paper together with all the authors.

Paper III

I designed the stability study together with my supervisors, Lars Nilsson, Marie Wahlgren, and Herje Schagerlöf. I designed the combination of techniques used to characterize excipient-induced stabilization and structural changes with Lars Nilsson and Marie Wahlgren. I developed the trypsin digestion method and the LC-MS method with Herje Schagerlöf. I performed the stability study, the water activity measurements, the trypsin digestion, the LC-MS experiments with Herje Schagerlöf, the DSC experiments with Amanda Västberg, and titration fluorescence spectroscopy with Johanna Hjalte. I also prepared samples for the NMR experiments. I analyzed and processed the LC-MS data, the NMR data with Carl Diehl, and the titration fluorescence spectroscopy data with Johanna Hjalte. I performed the structural analysis on GA-Z with an AlphaFold2-generated structure. I wrote the original draft and finalized the paper together with all the authors.

Paper IV

I developed the trypsin digestion method together with Ileana Rodríguez León and the LC-MS method with Herje Schagerlöf and Ileana Rodríguez León. I performed the AF4 experiments together with Mats Leeman and Alejandra Castro, the trypsin digestion, and the LC-MS experiments. I analyzed and processed the AF4 data with Lars Nilsson and the LC-MS data with Herje Schagerlöf and Ileana Rodríguez León. I wrote the original draft and finalized the paper together with all the authors.

Abbreviations

AF4	Asymmetrical Flow Field-Flow Fractionation
DSC	Differential scanning calorimetry
HMQC	Heteronuclear Multiple Quantum Coherence
HSA	Human serum albumin
LC	Liquid Chromatography
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
PBS	Phosphate Buffered Saline
RMSF	root mean square fluctuation
SANS	Small Angle Neutron Scattering
Tm	Denaturation temperature

Introduction

Research into the functions of proteins in the human body has greatly expanded our understanding of their diverse roles in various physiological processes. **Therapeutic proteins**, which mimic the actions of natural proteins, have revolutionized modern medicine. These proteins can regulate and target highly specific and complex biological processes, and therefore, treat a large variety of diseases. Therapeutic proteins are commonly used for treating e.g. cancer, autoimmune disorders, and chronic inflammatory diseases (1).

Five of the ten most sold drugs globally in, 2023 (revenue), were therapeutics proteins (2), and all these five are antibodies, the class of proteins that are most used as therapeutic proteins (2, 3). Other kinds of proteins used as therapeutic proteins are hormones, coagulation factors, enzymes, and fusion proteins (3, 4).

Therapeutic proteins differ from traditional small molecule drugs, being larger and more complex in their structure. They have both secondary and tertiary structures that affect their efficacy and function, and proteins are sensitive to chemical and physical degradation (5-8). Formulations of therapeutic proteins are developed to minimize degradation of the proteins. In liquid formulations, stabilizing polyols and surfactants hinder chemical degradation, aggregation, and denaturation (3, 9).

Aim

This doctoral thesis aims to investigate the stability of therapeutic proteins in solution. Herein, the following is examined: (1) How the structure of the affibody GA-Z affects its chemical stability. (2) How the excipients glycerol, fructose, sucrose, melezitose, polysorbate 80, and DDM influence the chemical degradation of GA-Z through deamidation and isomerization. (3) How the excipient-induced changes in chemical stability correlate to other excipient-induced changes in the thermal stability and the structure of GA-Z. This includes examining excipient-induced changes in solvent properties and their effect on the degradation of the residues in GA-Z. Finally, a novel *in vitro* methodology is developed to provide insight into the stability and behavior of therapeutic proteins in plasma *in vivo*. The purpose of the present work is to demonstrate that, after incubation in plasma, serum albumin binding can be investigated and quantified, changes in monoclonal antibody size can be analyzed, and monoclonal antibody aggregates can be identified and quantified.

The affibody GA-Z

GA-Z is an affibody containing two domains covalently connected by a linker. The domain at the N-terminal, the z-domain, binds to the protein Complement C5. Complement C5 is an inflammatory protein, part of the classical complement pathway, which is a defense system against bacterial and viral infections included in our immune system (10). The therapeutic function of GA-Z is its binding of Complement C5.

The domain at the C-terminal binds to albumin and is called the albumin-binding domain. The binding of human serum albumin (HSA) in blood prolongs the circulation time of GA-Z in the bloodstream after administration (11). Having two domains is characteristic of affibodies, a class of proteins of increasing therapeutic interest (12, 13). A structure of GA-Z is shown in Figure 1. Each domain consists of three α-helices, and there are no β-sheets. GA-Z is a small protein with a molecular weight of 11 865 Da and consists of 108 residues. In addition, its isoelectric point is approximately 4.5, and GA-Z is produced using E. coli and is, therefore, non-glycosylated (14). Importantly, GA-Z easily degrades chemically through deamidation and isomerization.

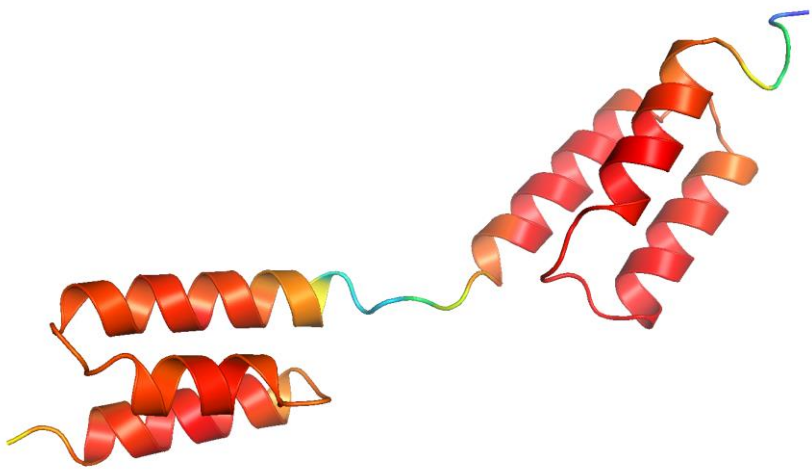

Figure 1. The structure of the affibody GA-Z. The structure is generated using AlphaFold2. It consists of two domains, each having three α-helices. The domains are covalently bonded by a linker.

Chemical degradation – Deamidation and isomerization

Chemical degradation change the chemistry of proteins and involve reactions between the atoms within the protein, between water and the protein, or between solutes and the protein. Examples of chemical degradation reactions are hydrolysis, reduction, racemization, oxidation, deamidation, and isomerization (15). Deamidation and isomerization are frequent in therapeutic proteins (5, 8). Since these reactions change the isoelectric point of the protein and the orientation of the peptide backbone of the degraded residues (8, 15), deamidation and isomerization may lead to decreases in the functionality and efficacy of therapeutic protein (5-8). Deamidation and isomerization can also cause aggregation (7, 16, 17) and immunogenicity in patients (5, 6, 8).

The deamidation and isomerization reactions are shown in Figure 2. Both reactions start with a nucleophilic attack from the nitrogen in the peptide backbone at the C-terminal side, against the carbon in the side chain carbonyl group of asparagine and aspartic acid residues. Before the attack, the nitrogen needs to be deprotonated by water. After the attack, either the amine or the hydroxylic group leaves the carbonyl center as ammonia or as water, and the side chains of asparagine and aspartic acid residues form a succinimide ring with the peptide backbone. The succinimide ring is in the L-form, however it might transfer to the D-form. The L- or D-succinimide ring then forms L- aspartic acid, L-iso-aspartic acid, D-aspartic acid, or D-iso-aspartic acid residues (18-23). Most of the residues are in L-form and the amount of iso-aspartic acid residues is 3-fold higher than aspartic acid residues (20-23). The rate of deamidation and isomerization depends on the rate of the deprotonation of the peptide backbone nitrogen and on the stability of the leaving group (18, 24). The physical properties of the solution, e.g. ionic strength and dielectric constant, impact the rate of the deprotonation of the nitrogen in the peptide backbone. The pH of the solution affects the charge of the leaving group and, hence, its propensity to leave. The charge of neighboring residues can also affect the rate of the deprotonation of the nitrogen in the peptide backbone. All these properties, therefore, influence the rate of deamidation and isomerization (18, 25-33).

Also, the shorter the distance between the deprotonated nitrogen and the carbonyl center carbon (the Cγ-N distance), the higher the propensity of deamidation and isomerization, and short distance increases both deamidation and isomerization (21, 24). The Cγ-N distance depends on the secondary and tertiary structures of a protein. Moreover, a high mobility of the peptide backbone increases the risk of obtaining conformations with a short Cγ-N distance. In the primary structure, the residues in the N+1 position influence the rate of deamidation and isomerization. A small residue in the N+1 position, e.g., glycine or serine, increases the mobility of the peptide backbone due to a limited steric hindrance, and hence deamidation and isomerization increase (18, 25, 28, 29, 34). Primary structures with a small residue

in the N+1 position to asparagine or aspartic acid residues are, therefore, referred to as “hot spots” for deamidation and isomerization. On the other hand, a bulky residue in the N+1 position, e.g., valine and leucine, lowers the rate of deamidation and isomerization. Secondary and tertiary structures often impose structural restrictions on the peptide backbone of asparagine and aspartic acid residues, and are known to lower the rate of degradation of “hot spot” residues (24, 30, 35-38). Moreover, in α-helices and β-turns, the hydrogen on the reactive peptide backbone nitrogen, is usually hydrogen bonding to neighboring residues, lowering the reactivity of the nitrogen.

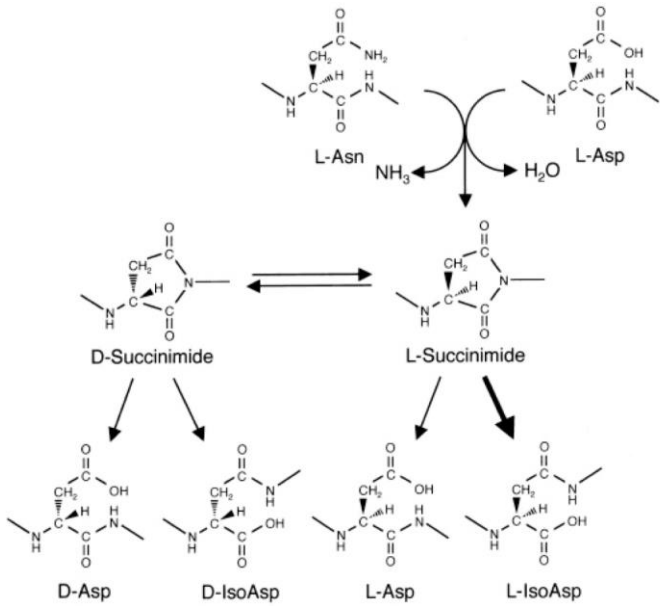

Figure 2. The deamidation and isomerization reactions. A simplified reaction scheme of the deamidation and isomerization reactions. The figure was created by Yang and Zubarev (22).

Physical degradation - Denaturation and aggregation

During physical degradation, the protein often loses its native structure. There are several forms of physical degradation processes. Proteins can denature, aggregate, form particles, precipitate, and adsorb to surfaces. In denaturation, the protein unfolds, and the protein loses its tertiary and secondary structures. The denaturation process is often reversible, and the protein can fold back to its native structure if the conditions promoting denaturation are removed. Denaturation and unstructured states of proteins may lead to aggregation, which leads to the formation of soluble and insoluble complexes of protein, where proteins have bound un-covalently or covalently to each other. The aggregation process is most often non-reversible and may lead to the formation of precipitated particles. Physical degradation commonly leads to a loss in protein function and may lead to immunogenicity in patients (8, 39-41).

Using excipients in liquid formulations of therapeutic proteins

Therapeutic proteins in liquid formulations must normally be stabilized against degradation. Therefore, each formulation is optimized by selecting a suitable buffer system and pH, and stabilizing excipients are often added. These stabilizing excipients often include surfactants and polyols (3, 9, 42).

Stabilizers – Glycerol and sugars

Polyols are molecules containing hydroxyl groups (−OH), such as glycerol (43, 44). Sugars also contain multiple hydroxyl groups (43). However, sugars are usually not referred to as polyols. In this study, the stabilizers glycerol and sugars will both be called polyols for simplicity. Figure 3 shows the structure of glycerol, fructose, sucrose, and melezitose. Polyols stabilize therapeutic proteins against aggregation and denaturation (45-53) and can stabilize the activity of enzymes (49, 54, 55). Glycerol and sucrose are often added to protein formulations (3, 9, 42), and in liquid formulations, glycerol is used at the concentration range of 1.3-55% v/v and sucrose at 25-200 mg/mL (3, 9, 42), corresponding to 1.5-12.4% v/v. The stabilizing effect of polyols is thought to be due to the preferential exclusion of the polyols from the protein surface (45, 56-59). Both experimental data and simulations show that polyols are excluded at concentrations up to 40% v/v, and are completely excluded from the first solvation shell (56, 57, 60-63). Above 40% v/v, experimental data and a simulation show that glycerol enters the solvation shell, and above 60% v/v, glycerol is not preferentially excluded from the first solvation shell (61, 64). The

exclusion costs energy and to compensate for the energy loss, the proteins minimize their contact area by reducing their volume (57, 58). Studies on polyols and proteins show that glycerol and sorbitol lower the partial specific volumes and increase the apparent adiabatic compressibility of proteins (57, 58, 64, 65). It is this polyol-induced shift toward the more compact state that explains the stabilizing effect of the polyols.

When it comes to the chemical stability of proteins, there are fewer studies on the effects of polyols. Nevertheless, previous studies on peptides have shown that glycerol decreases deamidation by decreasing the dielectric constant of the solvent, which lowers the rate of the deprotonation of the reactive nitrogen in the peptide backbone (18, 66). The sugars sucrose and trehalose reduce deamidation and isomerization of denatured lysozyme (53). Sucrose and mannitol also decrease the deamidation of peptides (47, 67, 68). In contrast, sucrose chemically destabilized a protein in an optimized formulation (69).

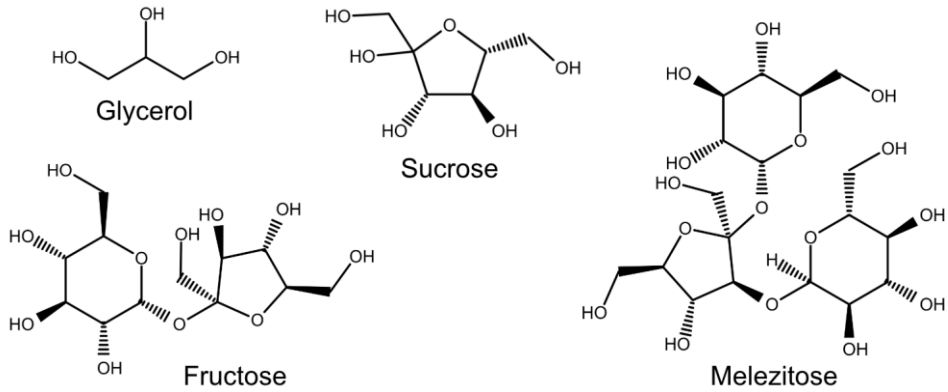

Figure 3. The structures of glycerol, sucrose, fructose, and melezitose. The polyols are used to stabilize proteins.

Non-ionic surfactants - Polysorbate 80 and Dodecyl-β-D-maltoside

Surfactants are amphiphilic molecules containing a hydrophobic part and a hydrophilic part. The hydrophilic part is referred to as the head-group, and the hydrophobic part is called the tail. Non-ionic surfactants have an uncharged head group (44). They are often used in liquid formulations of therapeutic proteins and are mainly used to inhibit aggregation, increase solubility, and lower the amount of surface adsorption (3, 9). In this context, the most common surfactant is polysorbate 80. Its critical micelle concentration is 0.013 mM, and it is used in the concentration range of 0.01-2 mg/mL (3, 9). The non-ionic surfactant n-Dodecyl-β-D-maltoside (DDM) is often used to solubilize membrane proteins (70) but has not been used in formulations of therapeutic proteins (3, 9). It has a critical micelle concentration of 0.17 mM in water (70). Very little is known about the effects of surfactants on the chemical stability of proteins. Figure 4 shows the molecular structures of polysorbate 80 and DDM.

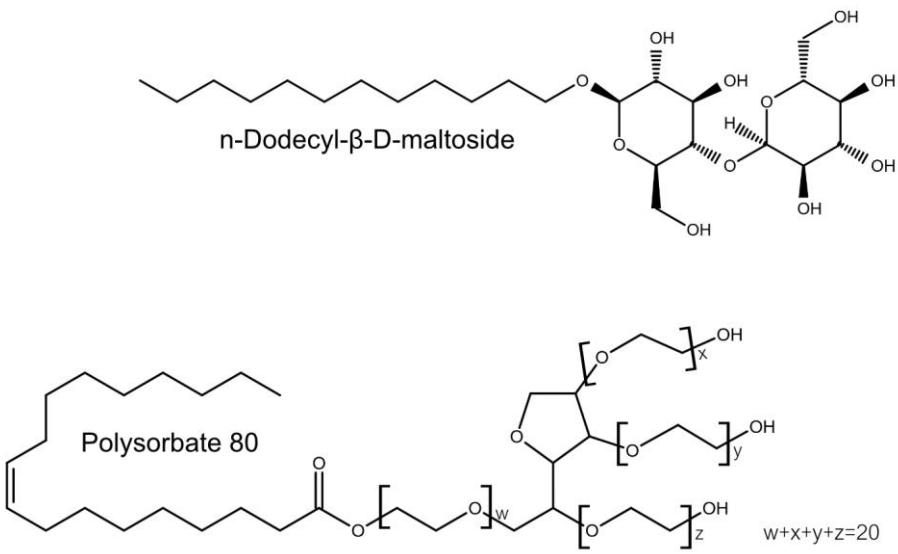

Figure 4. The structures of polysorbate 80 and DDM. The surfactants are used to stabilize and solubilize proteins.

The stability of therapeutic proteins in vivo

The stability of therapeutic proteins in their formulations is extensively examined and optimized (9, 39). However, their stability and general behavior *in vivo*, i.e. after administration, also need to be investigated, and immunogenicity, bioavailability, pharmacokinetic, and toxicokinetic studies are always conducted (71). Drugs of therapeutic proteins are mostly administrated through intravenous infusion or subcutaneous injection (3, 9, 39). After administration, the therapeutic proteins circulate in the bloodstream (72). The circulation times of therapeutic proteins vary, and the half-life ranges from a few hours up to weeks (73, 74). For antibodies, the half-life is often several weeks (75-77). Blood contains a plasma phase and a cellular phase (78, 79). The physicochemical and physical properties of blood, such as pH and ionic strength, often differ from those of the formulation. Therefore, proteins may deamidate, denature, precipitate, and aggregate in the bloodstream even if they are stable in their formulations. In addition, the fact that some therapeutic proteins circulate in the bloodstream for up to several weeks increases the risk of deamidation (75) and aggregation. Therapeutic proteins may also interact with blood components in ways that, are sometimes desirable and sometimes not. The *in vivo* studies mentioned above will detect most of these behaviors. However, *in vivo* studies are expensive, demanding, and require animal as well as patient studies. Therefore, new *in vitro* methods that pre-screen the behavior of proteins in plasma prior to *in vivo* analyses would be highly valuable and useful. Moreover, it may not be possible to characterize the binding of therapeutic proteins *in vivo*, since the formed complex may be altered by the sampling process.

The stability of GA-Z and its correlation to protein structure

The chemical stability of the affibody GA-Z was investigated by performing a stability study where the protein was stored for up to 41 days at 37 °C to accelerate the chemical degradation reactions. The degradation was then measured using Liquid Chromatography-UV (LC-UV) and Liquid Chromatography-Mass Spectrometry (LC-MS). LC-UV was used to determine the total amount of degradation after incubation, and LC-MS was used to identify the degradation products (Papers I-III). Figure 5 shows a Total Ion Chromatogram of degraded GA-Z obtained with LC-MS. After 41 days of incubation, 68% of GA-Z was degraded by hydrolysis, deamidation, and isomerization (Paper I).

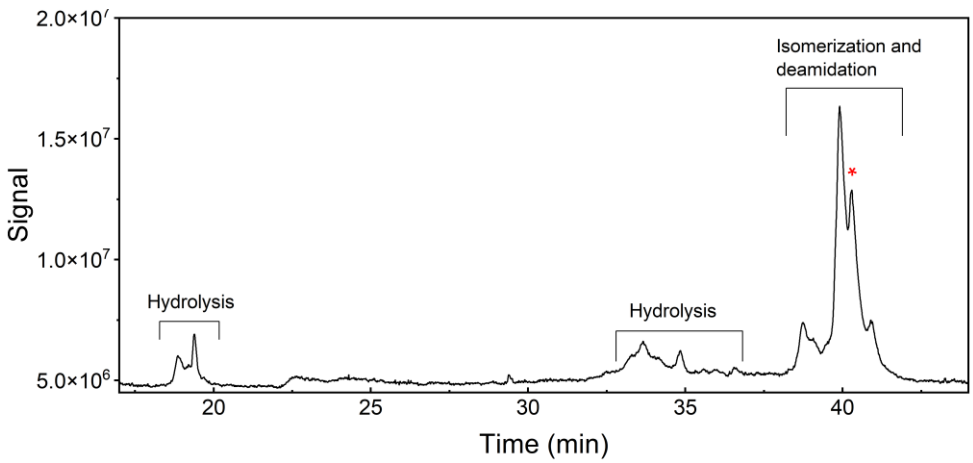

Figure 5. Total Ion Chromatogram of degraded GA-Z after 41 days of incubation at 37 °C obtained with LC-MS. The sample contains 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0), and GA-Z is degraded by deamidation, isomerization, and hydrolysis, as marked with brackets in the chromatogram. The peak containing native GA-Z is marked with an asterisk (*).

The degraded residues of GA-Z

To identify the deamidated and isomerized residues of GA-Z, the samples from the stability study were trypsin-digested into peptides, and the peptides were then analyzed with LC-MS. The residues Asp14, Asp17, Asn21, Asp35, Asp36, Asn52, Asp53, Asp60, Asn71, Asp75, and Asp81 deamidate or isomerize (Paper II). Moreover, it is concluded from the total ion chromatogram (Figure 5) that hydrolysis occurs at Asp37-Pro38, Asn52-Asp53, and Asp53-Ser54 (Paper I). Most of the degradation takes place in the z-domain and in the linker. Asn52 and Asp60 have the lowest chemical stability, and after 41 days of incubation, over 50% of Asn52 and Asp60 degrade (Paper II). An AlphaFold2-generated structure of GA-Z is used to investigate how the degradation of the residues depends on the structure of GA-Z, and it is shown that the structure highly impacts the propensity for degradation. Asparagine and aspartic acid residues with short Cγ-N distances, small or charged residues in the N+1 position, and high conformational flexibility of the peptide backbone increase deamidation and isomerization in GA-Z (Paper II). The result agrees with previous studies on how the structure of a protein affects deamidation and isomerization (18, 21, 23-25, 28-30, 34-38). Table 1 shows a summary of the deamidated and isomerized residues of GA-Z.

Table 1. The deamidated and isomerized residues of GA-Z. The table includes the amount of degradation, the Cγ-N distance, and the residue in the N+1 position of the different residues in GA-Z. The table also states in which domain the residues are positioned.

Residue	Degradation[a,b] (%)	Cγ-N distance (Å)	Structure	Residue N+1	Domain
Asp14	5.1/1.5 [a]	3.9	α-helix	Gln	z
Asp17	5.1/1.5 [a]	4.1	α-helix	Arg	z
Asn21	7.6 [a]	4.8	Loop	Leu	z
Asn32	0	4.7	α-helix	Lys	z
Asp35	20.5 [a]	4.1	α-helix	Asp	z
Asp36	2.8 [a]	4.0	Loop	Asp	z
Asp37	N/A	N/A	N/A	N/A	N/A
Asn52	53.8 [a]	3.6	α-helix	Asp	z
Asp53	7.4 [a]	4.7	α-helix	Ser	z
Asp60	54.0 [a]	4.8	Loop	Gly	Linker
Asn71	0.4[b]	4.7	α-helix	Ala	Albumin-binding
Asp75	0.8 [a]	4.7	α-helix	Ser	Albumin-binding
Asp81	0.3 [a]	4.8	Loop	Phe	Albumin-binding
Asp88	0	4.7	α-helix	Lys	Albumin-binding
Asp101	0	4.7	α-helix	Ala	Albumin-binding

[a] Degradation of the residues in GA-Z after 41 days of incubation at 37 °C.
[b] Degradation of the residues in GA-Z after 44 days of incubation at 37 °C.

The thermal stability of GA-Z

The thermal stability of GA-Z is investigated using Differential Scanning Calorimetry (DSC). Figure 6 shows the DSC thermogram of GA-Z, which displays two peaks corresponding to two energy transitions of the protein. The first peak corresponds to the denaturation of the z-domain, with unfolding beginning at approximately 26 °C. At 40 °C (Tm_1), 50% of the z-domain population is in an unfolded state. The second peak corresponds to the denaturation of the albumin-binding domain, with the onset of denaturation overlapping with the first transition peak. The denaturation temperature for the albumin-binding domain (Tm_2) is 66 °C. The DSC thermogram shows that the z-domain is more thermally unstable than the albumin-binding domain. Additionally, the Tm_1 of the z-domain is close to the storage temperature of GA-Z in the stability study (37 °C), which means that approximately 50% of the z-domain population was in an unfolded state during storage. The unfolded state of the protein significantly increases the conformational flexibility of the peptide backbone, thereby enhancing the propensity for deamidation and isomerization (18, 20, 21, 27, 32, 35). Therefore, the high amount of chemical degradation in the z-domain is likely caused by the unfolded state observed in our study (Paper II).

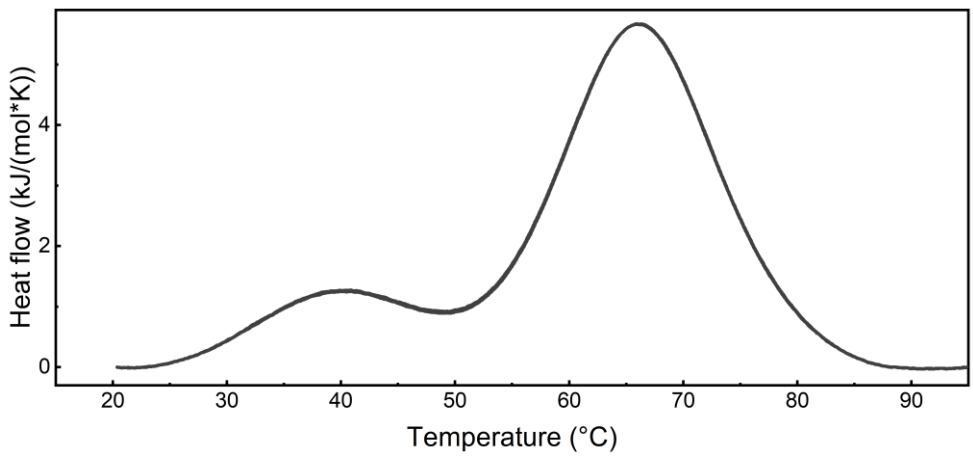

Figure 6. The DSC thermogram of GA-Z. The sample contains 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0). The measurements are performed in duplicate and are included in the figure.

The effects of polyols on the stability and structure of GA-Z and its correlation to structural changes

The effect of the polyols glycerol and three sugars (fructose, sucrose, and melezitose) on the chemical stability of GA-Z was investigated. GA-Z in 0-90% v/v glycerol and 0-30% v/v fructose, sucrose, and melezitose was stored at 37 °C for up to 41 or 44 days and then analyzed with LC-UV and LC-MS. LC-UV was used to determine the total amount of degradation after incubation. LC-MS was used to measure the degradation of various residues and was performed on trypsin-digested GA-Z. All the polyols were shown to increase the chemical stability of GA-Z (Paper I-III). Adding 30% v/v glycerol lowers the total amount of degradation of GA-Z from 68% to 52%, and increasing the glycerol concentration to 90% v/v further reduces the degradation to 36% (Paper I). Glycerol and sugars affect the chemical stability of GA-Z in similar ways. The effect of polyols on the chemical stability of the residues in GA-Z depends on the specific residue's local structure and type. The isomerization of aspartic acid residues in α-helices decreases linearly with increasing polyol concentrations, and the deamidation of asparagine residues also decreases linearly with increasing polyol concentrations, independently of their local structures. At 70% v/v glycerol, the deamidation and isomerization of most of the aspartic acid and asparagine residues in α-helices reach zero. In contrast, when increasing the concentration of polyols from 0 to 50% v/v the isomerization of Asp60 in the linker increases. However, the isomerization of Asp60 decreases when adding more than 50% v/v glycerol, and at 90% v/v glycerol, the amount of isomerization of Asp60 is lower than at 0% v/v glycerol. Moreover, some variations in the stabilizing effects of glycerol and sugars were observed. Glycerol destabilizes the aspartic acid residues in loops, as seen for Asp60. However, these residues are stabilized when adding sucrose and melezitose. In general, the two domains of GA-Z are affected equally by the polyols, except that degradation increases in the albumin-binding domain when adding more than 50% v/v glycerol, which was not observed in the z-domain (Papers II and III). The effect of fructose on the stability of GA-Z differs slightly from that of sucrose and melezitose. However, fructose is a reducing sugar (44), which induces chemical reactions with GA-Z. These reactions may have altered the chemical composition of GA-Z, thereby also changing the

composition of the GA-Z peptides analyzed with LC-MS, and the data on the chemical stability of GA-Z in fructose may be less accurate (Paper III).

Glycerol-induced changes of the exchange rate between the L-succinimide and the D-succinimide rings

Figure 7 shows the amount of Asp60 isomerization. The isomerization of Asp60 leads to the formation of three isomerization products: L-isoAsp60, D-isoAsp60, and D-Asp60. The isomerization products are identified by comparing the rates of formation of the three products. The L-form is known to have a higher formation rate than the D-forms, and iso-Asp is formed at a higher rate than Asp (20-23). Figure 2 shows a simplified reaction scheme of the isomerization reactions and shows how the three products are formed. At 0% v/v glycerol, 48% of the isomerized Asp60 residues form L-isoAsp60, 4% form D-isoAsp60, and 1% form D-Asp60.

Adding glycerol to GA-Z increases the formation of all isomerization products. However, the D-forms increase more than the L-form. At 50% v/v glycerol, where the isomerization of Asp60 was the highest, the formation of L-isoAsp60 only increased by 1%. D-isoAsp60 increased by 5% and D-Asp by 3%. Moreover, D-isoAsp and D-Asp more than doubled in number. The results show that glycerol shifts the kinetics of the exchange rate between the L-succinimide ring and the D-succinimide ring toward a faster formation of the D-succinimide ring. This shift may be caused by a glycerol-induced change in solvent properties, e.g. a change in dielectric constant.

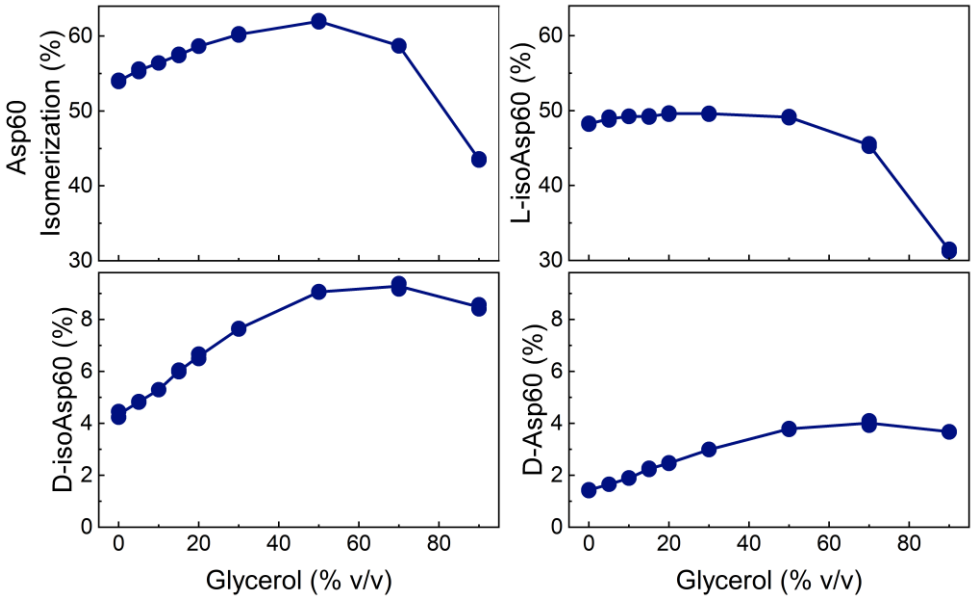

Figure 7. Isomerization of Asp60 after 41 days of incubation at 37 °C in 0-90% v/v glycerol. The isomerization of Asp60 produces the three products L-isoAsp60, D-isoAsp60, and D-Asp60. All stability samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0).

Polyols-induced changes in the solvent properties

The physical properties of the solvent are known to affect the rate of deamidation and isomerization (18, 26-33). pH and ionic strength are kept constant in the stability studies (Papers I-III). However, adding polyols lowers the water activity and the dielectric constant of the stability samples (18, 80). Figure 8 shows how the polyol concentration affects solvent properties using two measures. The left axis shows the water activity of the stability samples containing polyols (Papers I and III), and the right axis shows the dielectric constant at 0-80% v/v glycerol (18). The water activity and the dielectric constant affect the deprotonation rate of the peptide backbone nitrogen, which, in turn, initiates deamidation and isomerization. Hence, the water activity and the dielectric constant, also affect the rate of deamidation and isomerization (18). Between 0-30% v/v polyols, the water activity is high, close to 1, and has no effect on the chemical stability of GA-Z. However, at higher concentrations, above 30% v/v, the water activity decreases, which likely stabilizes GA-Z against chemical degradation. The dielectric constant decreases at 20% v/v glycerol and might reduce the degradation of asparagine residues at lower polyol concentrations (< 30 % v/v).

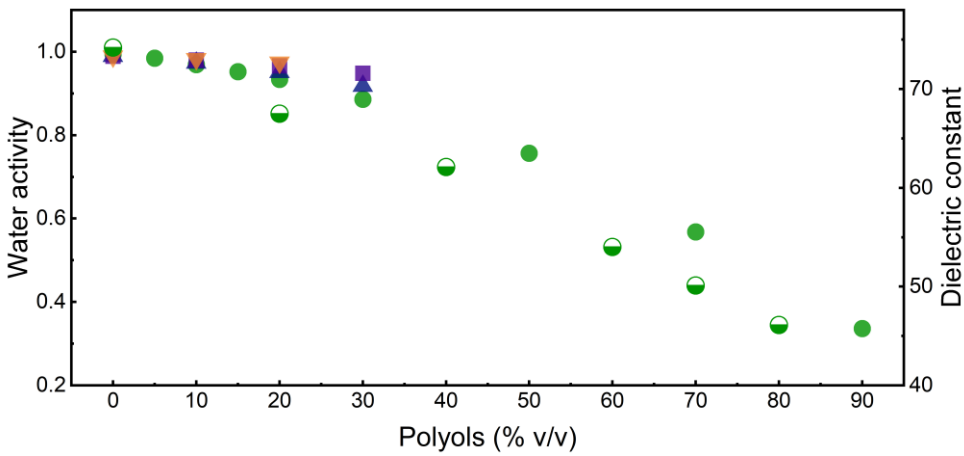

Figure 8. Solvent properties of the stability samples. The water activity of the stability samples with 0-90% v/v glycerol (●), 0-30% v/v fructose (▲), sucrose (■), and melezitose (▼). All stability samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0). The figure also shows the dielectric constant at 0-80% glycerol v/v (◒) (18).

How variations in the dielectric constant affect isomerization in unstructured regions of GA-Z

The Asp60 position in the linker of GA-Z is destabilized by glycerol, fructose, and sucrose. Asp36 and Asp81, which are located in loops, are destabilized by glycerol (Papers II and III). The lowering of the dielectric constant (Figure 10) increases the pK_a of the carboxylic groups in the side chains of the aspartic acid residues (18). An increase in the pK_a increases the number of uncharged carboxylic groups, which in turn increases the propensity for the hydroxylic group to leave as water, promoting isomerization (18). The linker and the loops of GA-Z lack higher-order structures. The residues located in the linker and in the loops are, therefore, less protected against deamidation and isomerization (21, 23, 24, 30, 35, 38). This lack of higher-order structures makes the stability of residues in the loops and the linker more dependent on solvent properties, such as the dielectric constant, than the residues located in the ordered α-helices. Therefore, the polyol-induced destabilization of Asp60, Asp36, and Asp81 is probably caused by the decrease in the dielectric constant of the solvent.

The impact of polyols on the thermal stability of GA-Z

The thermal stability of GA-Z in the presence of polyols was investigated using DSC (Papers II and III). The denaturation temperatures, Tm_1 and Tm_2, in 0-30% v/v polyols were determined using the obtained DSC thermograms and are shown in Figure 11. All polyols increase Tm_1 and Tm_2 linearly. Our findings regarding the polyol-induced stabilization of GA-Z agree with several studies that show that glycerol and sugars increase the thermal stability of proteins (46, 49-52). Fructose, sucrose, and melezitose all show the same increase of Tm_1 and Tm_2. Here, the concentrations of the sugars are compared by their volume fractions and not by weight or molarity. The stabilizing effect of polyols on the thermal stability of proteins is proposed to be due to the preferential exclusion of polyols from the surface of the protein (45, 56-59). The equal stabilizing effect observed when comparing the volume fractions of the various sugars indicates that it is the excluded volumes of the sugars that affect the level of stabilization.

A small difference is observed in the increase in Tm_1 when adding glycerol compared to adding sugars at equivalent concentrations. When increasing the polyol concentrations from 0 to 30% v/v, glycerol increases Tm_1 by 3 °C more than sucrose. The inverse effect is observed for Tm_2, where an increase in the sucrose concentration to 30% v/v increases Tm_2 by 9 °C more than the equivalent increase of the glycerol concentration. The larger stabilizing effect of sucrose on the albumin-binding domain could correlate to a more effective exclusion of sugars

from the surface of the albumin-binding domain compared to glycerol. The chemical and physical properties of sugar and glycerol vary, and their differences may affect how efficiently they are excluded from the surface of GA-Z.

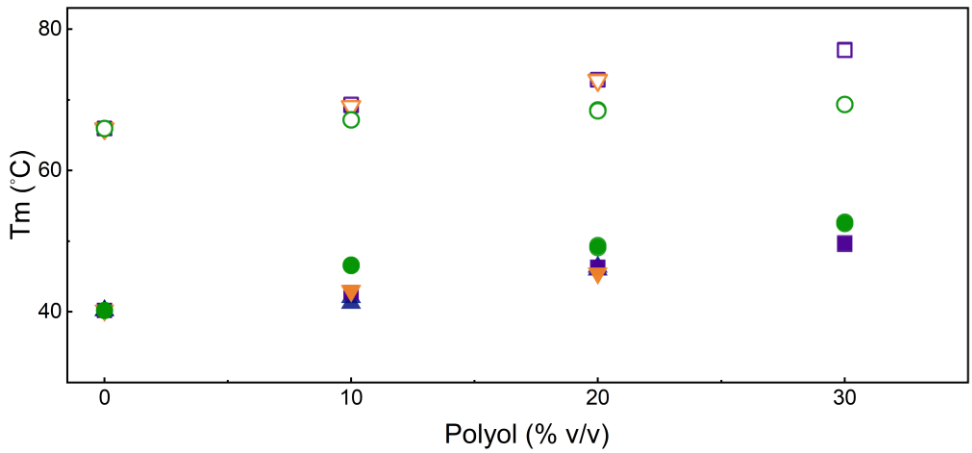

Figure 9. Polyol-induced changes in the denaturation temperatures of GA-Z. Tm_1 of GA-Z in glycerol (●), fructose (▲), sucrose (■), and melezitose (▼) and Tm_2 of GA-Z in glycerol (○), sucrose (□), and melezitose (▽) vs polyol concentration (% v/v). The samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0).

Polyol-induced structural changes of GA-Z

Polyol-induced conformational changes of GA-Z were investigated using Asymmetrical Flow Field-Flow Fractionation (AF4), Small Angle Neutron Scattering (SANS), and two-dimensional Nuclear Magnetic Resonance (NMR) spectroscopy (Papers I-III).

Elongation of GA-Z

AF4 was used to analyze the size of GA-Z in 0-9% v/v (Paper I). The experiment was performed at 20 °C, and GA-Z was, therefore, in a folded state, having an onset for denaturation at 26 °C (Figure 6). By using the AF4 elution times, the hydrodynamic radius of GA-Z was calculated. At 0% v/v glycerol, the hydrodynamic radius is 35 Å, and at 9% v/v glycerol, the hydrodynamic radius increases to 42 Å. MALS-RI measurements on the AF4 eluent were used to determine the molecular weight of GA-Z. The molecular weight is 11 000 ± 400 Da over the entire AF4 peak at all glycerol concentrations. The mass corresponds to the molecular weight of the monomer of GA-Z (11 865 Da). The two domains of GA-

Z are able to move toward or away from each other. When the domains are further apart, GA-Z is elongated, and the hydrodynamic radius increases, as indicated above

GA-Z was also examined in 0-90% v/v glycerol-d_8 using SANS (Paper I). The SANS experiments were also performed at 20 °C, and GA-Z was in a folded state. From the SANS measurements, the p(r) functions were calculated. The p(r) function is a histogram of all possible distances within the structure of the protein. The p(r) function of GA-Z shows two distinct peaks at approximately 16 and 40 Å. The first peak results from the scattering within each of the two domains (81, 82) and shows the dimensions of the domains. The second peak results from the scattering between the two domains and, therefore, the distance between the two domains (81, 82). As the domains move around the linker, the protein is able to obtain multiple conformations, and the distances between the domains, represented by the second peak, show the ensemble of the conformations that GA-Z obtains. When glycerol is added, the r values of the second peak increase, showing that the average distance between the domains increases. In the absence of glycerol, the average distance between the domains is 38.7 Å, and the distance increases to 41.4 Å at 70% v/v glycerol. Both AF4 and SANS, therefore, indicate that glycerol induces more elongated conformations of GA-Z.

Compaction of the domains of GA-Z

Figure 10 shows the p(r) functions of GA-Z, which represents the dimensions of the domains, zoomed in on the first peak at glycerol concentrations ranging from 0-90% v/v. By analyzing the r values of the first peak of the p(r) function, it is observed that the domains become smaller when adding glycerol, and the r value decreases from 15.3 Å to 14.4 Å as the glycerol concentration increases from 0 to 90% v/v. This decrease in the volume of the domains is consistent with studies showing that glycerol lowers the partial specific volumes and increases the apparent adiabatic compressibility of proteins (57, 58, 64, 65).

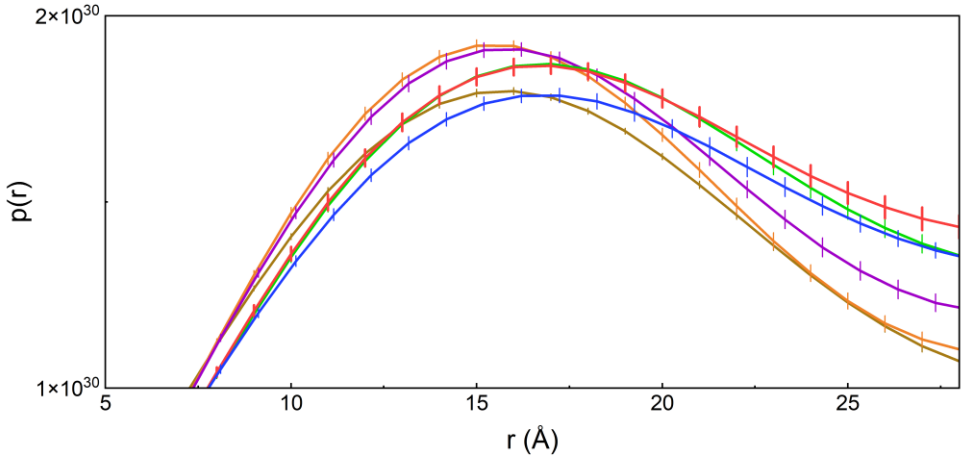

Figure 10. The p(r) functions of GA-Z, which represent the dimensions of the domains, zoomed in on the first peak. The p(r) functions were calculated from SANS data obtained at 20 °C. All samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0) and 0% (—), 5% (—), 30% (—), 50% (—), 70% (—), or 90% (—) v/v glycerol-d_8.

Stabilizing the folded state of GA-Z

The polyol-induced stabilization of the folded state of GA-Z was investigated using two-dimensional NMR spectroscopy (Papers II and III). HMQC spectra of GA-Z in 0-30% v/v glycerol, fructose, sucrose, and melezitose were recorded at 37 °C, and GA-Z was, partly unfolded in the absence of polyols (Figure 6). Adding polyols increases the denaturation temperature of Tm_1, thereby shifting the z-domain toward a folded state. The effect that shifting GA-Z toward its folded state may have on its residues was investigated by determining how the polyols affect the HMQC peak intensities (Paper II). Figure 11 shows how glycerol and sucrose affect the z-domain and the albumin-binding domain. Fructose and melezitose are not included in Figure 11, since they and sucrose yield similar NMR data of GA-Z. In the figure, the NMR-assigned residues are colored from green to red. Colors from green to yellow indicate that the environments of residues do not shift toward a more ordered state when adding the polyols, and the colors from orange to red show that the environments of the residues shift toward a more ordered state in the presence of polyols. The environments of the residues Leu28, Glu47, and Lys49 on the surface of the z-domain shift toward a more ordered state when adding 0-30% v/v glycerol (Figure 11a). The environments of the residues Ile16 and Leu34 in the core of the z-domain do not shift toward a more ordered state when adding glycerol. The glycerol-induced stabilization of the folded state of the z-domain is similar to the stabilization of the folded state of the domain obtained by decreasing the temperature to 22 °C. Glycerol has a low effect on the HMQC peak intensities of the residues Leu74, Lys 84, Ile103, Leu104, and Leu107 (Figure 11b), which shows

that glycerol does not fold the albumin-binding domain. The results agree with the DSC measurements, showing that at 37 °C, the z-domain gradually shifts toward the folded state when adding glycerol and that the albumin-binding domain already is folded at 0% glycerol (Paper II).

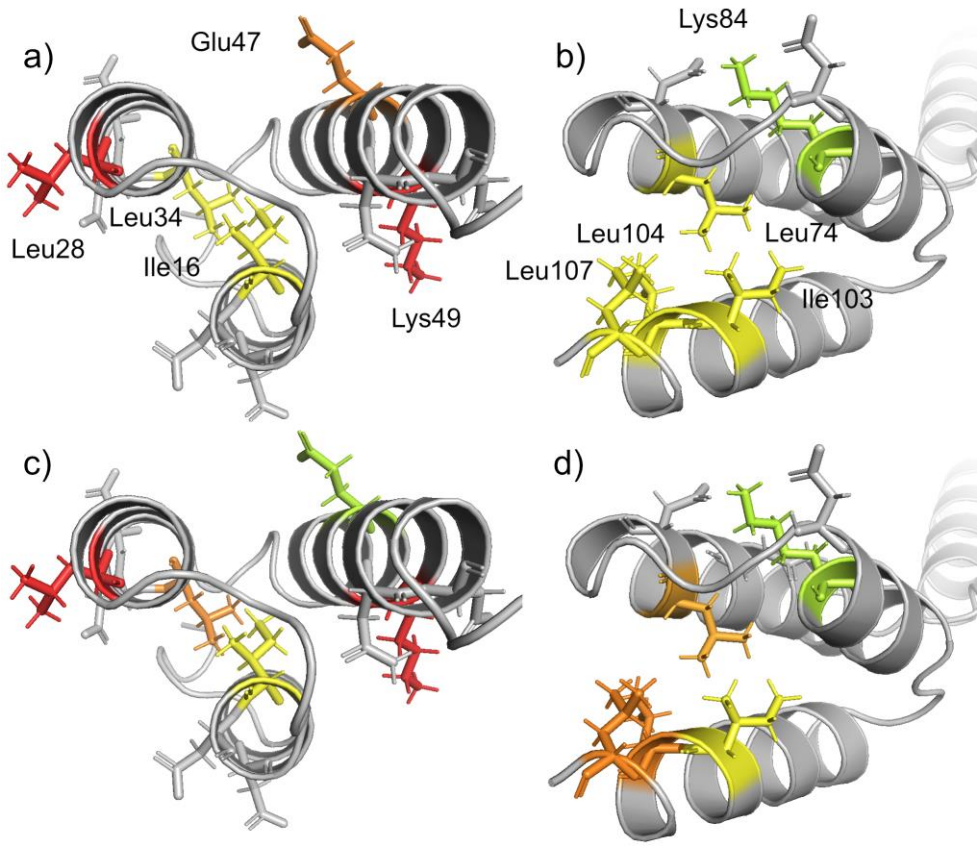

Figure 11. The effect of stabilizing the folded state of GA-Z on its residues. The analysis is performed by examining the HMQC peak intensities in spectra of GA-Z in 0-30% v/v polyols. a) How the residues in the z-domain are affected by glycerol, b) how the residues in the albumin-binding domain are affected by glycerol, c) how the residues in the z-domain are affected by sucrose, and d) how the residues in the albumin-binding domain are affected by sucrose. The colors from green to yellow show that the environments of the residues of GA-Z do not shift toward a more ordered state when adding polyols. The colors from orange to red show that the environments of the residues shift toward a more ordered state when adding polyols. All samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0).

When adding 0-30% v/v sucrose, the environments of the residues Leu28 and Lys49 shift toward a more ordered state (Figure 11c). However, in contrast to glycerol, the environment of Glu47 does not undergo the same shift. Chemical shift data of Glu47 show that sucrose changes its chemical shift, showing that the chemical environment of the residue alters, indicating that sucrose changes the structure of Glu47 (Paper III). Other factors than folding can influence the HMQC peak intensities (Paper II), and the chemical shift change might indicate that sucrose shifts the environment of Glu47 toward a more ordered state, even though the intensity data suggests the opposite. Moreover, at 37 °C and 30% v/v sucrose, the z-domain is not completely in a folded state (Figure 6), and the concentration of sucrose may need to be increased to stabilize the folded state of Glu47. The intensity of the peak corresponding to the residue Leu34 is slightly affected by sucrose, which may show that the core of the z-domain is in a more folded state in the presence of sucrose than it is in the presence of glycerol. In the albumin-binding domain, the addition of sucrose also shifts the environments of the residues Leu74, Leu104, and Leu107 toward a more ordered state (Figure 11d). The shift is hypothesized to be related to an extension of α-helix 3 to include the residue Leu107, which is situated in a loop connected to the α-helix and the C-terminal (Paper III). The shift of the environments of the residues Leu74, Leu104, and Leu107 can also be obtained by lowering the temperature to 22 °C (Paper II). The sugars increase Tm_2 more than glycerol does (Figure 9), and the sugar-induced shift of the area toward a more ordered state may be related to the higher increase of Tm_2 obtained when sucrose is added.

Structural changes of the albumin-binding domain

The two-dimensional NMR data were also used to investigate structural changes of GA-Z in 0-30% v/v glycerol, fructose, sucrose, and melezitose. Figure 12 shows how 0-30% glycerol and sucrose change the chemical shifts of the assigned peaks corresponding to residues in the albumin-binding domain. Glycerol changes the chemical shifts of all residues (Figure 12a). The chemical shift changes confirm that the chemical environments of the residues change when adding glycerol, which suggests that the structures of the residues are altered. As shown by the denaturation temperatures of GA-Z (Figure 9) and the HMQC peak intensities (Figure 12), the albumin-binding domain is in a folded state at 0% v/v polyols, and the observed shift changes are, therefore, not related to a stabilization of the folded state. Instead, the chemical shift changes likely indicate that glycerol induces a minor structural change in the albumin-binding domain. As glycerol, sucrose, changes the chemical shifts of most of the residues in the albumin-binding domain (Figure 12b), indicating that it too induces a small structural change of the domain. In addition, sucrose induces a larger change in the chemical shifts of Ile103 and Leu104 compared to

glycerol. The larger change in the chemical shifts of Ile103 and Leu104 may be due to the hypothesized extension of α-helix 3.

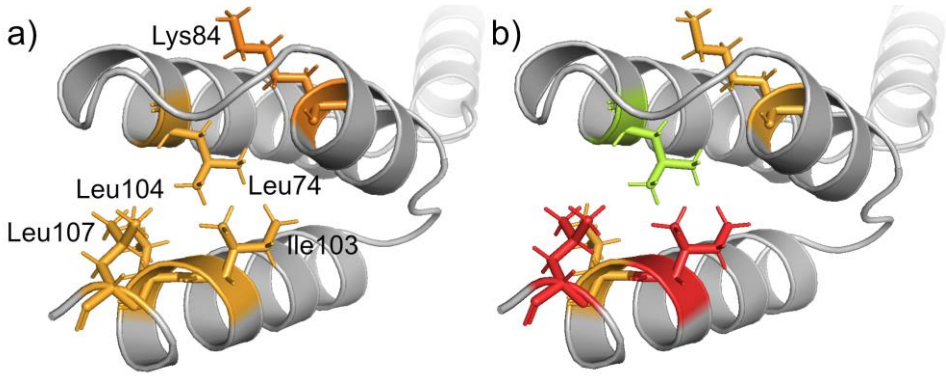

Figure 12. Polyol-induced structural changes of the residues in the albumin-binding domain. The analysis is performed by examining the chemical shifts of the assigned HMQC peaks in spectra of GA-Z in 0-30% v/v polyols. a) Residues in the albumin-binding domain that exhibit chemical shift changes upon the addition of glycerol and b) residues in the albumin-binding domain that exhibit chemical shift changes upon the addition of sucrose. The colors from green to yellow show that the chemical environments of the residues do not change when adding polyols. The colors from orange to red show that the chemical environments of the residues change when adding polyols. All samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0).

The chemical shifts of the assigned residues in the NOESY spectra of GA-Z in 0-30% v/v glycerol showed that glycerol changed the H_α-shifts of the residues in the albumin-binding domain (Tabel 2, Paper II). The H_α-shifts move upfield in the spectra, and since the assigned H_α shifts are located in or adjacent to α-helices, the observed shift changes show that the α-helical population is stabilized by glycerol (83). The stabilization of the α-helical population results in GA-Z shifting toward a more ordered state, likely making its structure more rigid. The shift toward the more ordered state is likely the source of the small structural change observed in the HMQC spectra (Figure 12, Papers II and III). The polyol-induced small structural change of the albumin-binding domain is in agreement with several studies that have shown that glycerol generates minor conformational changes in the secondary and tertiary structures of proteins (55, 64, 84, 85).

Table 2. The chemical shift changes in the albumin-binding domain for $^1H_\alpha$ when adding 0-30% v/v glycerol-d_8.

Structure	Residue	$\Delta\delta$-$^1H_\alpha$
α-helix 1	Leu74	-0.031
α-helix 2	Lys84	-0.030
Loop	Thr92	-0.037
α-helix 3	Lys100	-0.036
α-helix 3	Ile103	-0.033
α-helix 3	Ala105	-0.036

Preferential exclusion of polyols and structural changes of GA-Z

The SANS data indicate that polyols induce a compaction of the domains (Figure 10). This compaction is likely correlated to the minor structural change of the albumin-binding domain, observed with NMR spectroscopy upon the addition of 0-30% polyols (Figure 12, Table 2, Papers II and III). Moreover, it is likely that the same type of structural change that occurs in the albumin-binding domain also occurs in the z-domain when the domain is in a folded state (>30% v/v polyols). Between 0-30% v/v glycerol, the compaction of the domains is not observed in the SANS data. However, NMR spectroscopy has a higher resolution than SANS (86, 87) and may make it possible to observe the polyol-induced structural changes of GA-Z before they are large enough to be detected by SANS. When glycerol is added, the α-helical population is stabilized, and the albumin-binding domain shifts toward a more ordered state (Table 2), which likely decreases the volume of the domain, as seen in the SANS data. It has been shown that the preferential exclusion of sorbitol decreases the root mean square fluctuation (RMSF) of the C_α-atoms in trypsin (55). The decrease in the RMSF values shows that the conformational flexibility of the peptide backbone of trypsin decreases. The observed structural change may be similar to the glycerol-induced structural change of the H_α-atoms in the albumin-binding domain, as both the observed changes in trypsin and the glycerol-induced change in the H_α-atoms lower the conformational flexibility of the peptide backbone. Moreover, studies have shown that the preferential exclusion of glycerol leads to a decrease in the partial specific volumes of proteins (57, 58). It is, therefore, likely that the structural change of the albumin-binding domain, correlated to the observed compaction of the domains, is a consequence of the preferential exclusion of the added polyols. Furthermore, it has also been shown that the preferential exclusion of polyols folds (63) and increases the structural transition temperatures (Tm) of proteins (61). Therefore, the stabilization of the folded state

of the z-domain and the minor structural change of the albumin-binding domain are, likely caused by the addition of polyols and their subsequent preferential exclusion.

The exclusion of the polyols from the surface of GA-Z most likely costs energy. Therefore, GA-Z minimizes its contact area by reducing its volume in order to compensate for the energy loss, as previously suggested for other proteins (57, 58). The reduction of the area of GA-Z stabilizes the folded state of the z-domain and decreases the volumes of the domains by inducing a small conformational change toward a more ordered state.

The effects of polyols on the chemical stability of GA-Z

As shown in this study, polyols change the solvent properties, stabilize the folded state of the z-domain, and induce a small structural change of GA-Z, all of which potentially affect the chemical stability of GA-Z.

Polyol-induced stabilization of the z-domain at concentrations ranging from 0 to 30% v/v.

As previously stated, GA-Z mostly degraded in the z-domain, and the degradation of the z-domain was probably high, as it was partly unfolded during the stability study (Figure 6). An unfolded state increases the conformational flexibility of the peptide backbone, which, in turn increases the propensity for deamidation and isomerization (21, 23, 24, 30, 35, 38). Adding polyols stabilizes the folded state of the z-domain, as shown by DSC and NMR spectroscopy (Figures 9 and 11). This stabilization leads to a decrease in the conformational flexibility of the peptide backbone, which reduces the degradation of the asparagine residues at all positions and aspartic acid residues in α-helices. At 30% v/v polyols, the z-domain is close to fully folded (Papers II and III), and the residues located in α-helices show almost a halving of their degradation. Asp36, situated in a loop, is destabilized by glycerol (Paper II). However, it is slightly stabilized by sucrose and melezitose (Paper III). The glycerol-induced destabilization is, as previously mentioned, attributed to the lowering of the dielectric constant of the solvent (18). The sugar-induced stabilization shows that sugars may be able to reduce the conformational flexibility of the peptide backbone of Asp36. Moreover, the residue Asn21, also positioned in a loop, is highly stabilized by glycerol, fructose, sucrose, and melezitose. This residue is located close to Leu19, in the same loop. NMR data shows that Leu19 is part of an area of GA-Z that shifts toward a folded state upon the addition of 0-30% v/v glycerol (Paper II), and the stabilization of Asn21 could, therefore, be the result of a polyol-induced stabilization of the folded state of the z-domain.

Polyol-induced stabilization of the albumin-binding domain

In the albumin-binding domain, the residues Asn71, Asp75, and Asp81 degrade over time. The addition of 0-50% v/v polyols stabilizes Asn71 and Asp75, which are located in α-helices (Papers II and III). However, the albumin-binding domain is in a folded state at 0% v/v polyols (Figure 5). Chemical shift data of the assigned residues in the albumin-binding domain indicate that the polyols induce a small structural change of the domain (Figure 12, Table 2), in which the α-helices of the albumin-binding domain shift toward a more ordered state (Table 2). The more ordered state reduces the conformational flexibility of the peptide backbone and, therefore, likely is responsible for the observed increase in chemical stability of Asn71 and Asp75. Asp81, positioned in a loop, is destabilized by glycerol and stabilized by fructose, sucrose, and melezitose as is Asp36 (Papers II and III). The glycerol-induced destabilization is, as for Asp36, attributed to the lowering of the dielectric constant of the solvent (18) (Paper II). Asp36 and Asp81 are situated adjacent to α-helices. As mentioned, it is from the NMR data hypothesized that α-helix-3 in the albumin-binding domain is extended to include parts of the loop connected to the C-terminal upon adding 0-30% v/v sugars (Paper III). It is possible that the α-helices adjacent to Asp36 and Asp81 also are extended to include the residues Asp36 and Asp81 when sugar is added. The structural change would lower the conformational flexibility of the peptide backbone of Asp36 and Asp81 and, hence, lead to the chemical stabilization of Asp36 and Asp81.

SANS measurements show that the domains of GA-Z decrease in volume and become more compact when adding 50-90% v/v glycerol (Figure 10, Paper I). Interestingly, the isomerization of Asp75 and Asp81 increases at glycerol concentrations of 50-90% v/v. The increased degradation is not observed for the residues in the z-domain. The reduced volume of the albumin-binding domain could lower the Cγ-N distances of Asp75 and Asp81, which could explain the increased degradation. It is, however, not clear why the compaction would only lower the Cγ-N distances for the residues in the albumin-binding domain and not in the residues in the z-domain.

Glycerol-induced stabilization of the z-domain at concentrations above 30% v/v.

At 30% v/v glycerol, where the z-domain is close to fully folded, all residues that degrade in the absence of glycerol still degrade. By increasing the concentration of glycerol to 90% v/v, the degradation of all residues reached zero. It can be concluded that the chemically stabilizing effect of glycerol is not due to the stabilization of the folded state at concentrations above 30% v/v. As previously stated, it is likely that the glycerol-induced small structural change of the albumin-binding domain, shifting the domain toward a more ordered state, also occurs in the z-domain when

the z-domain is in a folded state (>30% v/v glycerol). Hence, the glycerol-induced increase in the chemical stability of the residues in the z-domain at concentrations above 30% v/v is likely due to the glycerol-induced small structural change (Paper II).

The effect of solvent properties on the chemical stability of GA-Z

The water activity of the stability samples in 0-30% v/v polyols is high (Figure 8) and does not influence the extent of deamidation and isomerization. However, above 30% v/v polyols, the water activity decreases, which may lead to reduced levels of deamidation and isomerization in the z-domain, in the albumin-binding domain, and in the linker. As the glycerol concentration increases to 50-90% v/v, the degradation of Asp36 and Asp60 decreases (Paper II). This decrease could be attributed to the reduction in water activity caused by high glycerol concentrations, as the stability of these residues is more dependent on solvent properties. The dielectric constant decreases at glycerol concentrations above 20% v/v (Figure 8), which may also contribute to the reduction in deamidation observed between 20-90% v/v glycerol.

The effect of polyols on the chemical stability of the linker

As previously mentioned, Asp60 in the linker is highly isomerized after 41 days of incubation (Papers II and III). The high level of isomerization is correlated to the high conformational flexibility of the peptide backbone in the linker (Paper II). The isomerization of Asp60 increases at glycerol concentrations of 0-50% v/v (Figure 7). The increase is attributed to the lowering of the dielectric constant of the solvent (18) (Paper II). The AF4 and SANS investigations show that glycerol induces a more elongated conformation of GA-Z between 0-50% v/v glycerol (Paper I). This elongation ought to affect the structure of the linker. The destabilization of Asp60 may, therefore, also be due to the structural change of the linker, if this change generates a local structure of Asp60 with a higher propensity for degradation. However, since the conformational flexibility of the linker seems to remain high in its elongated form, it is more likely that the destabilization of Asp60 is caused by the lowering of the dielectric constant of the solvent.

Stabilization through the preferential exclusion of polyols

Since the polyol-induced stabilization of the folded state of the z-domain and the small structural change of the albumin-binding domain most likely result from the preferential exclusion of polyols, and the polyol-induced chemical stabilization is caused by these changes, it can be concluded that polyols enhance the chemical stability of GA-Z by being preferentially excluded from the surface of GA-Z.

The effect of non-ionic surfactants on the stability of GA-Z and its correlation to structural changes

The effect of the non-ionic surfactants DDM and polysorbate 80 on the chemical stability of GA-Z was investigated in a stability study, where GA-Z in 0-0.8 mM polysorbate 80 and in 0-10.9 mM DDM was stored at 37 °C for 0-44 days. The amount of deamidation and isomerization of GA-Z in the stability samples was measured using LC-MS. The stability samples were trypsin-digested before the analysis. The LC-MS investigation shows that polysorbate 80 has a minimal impact on the chemical stability of GA-Z. DDM reduces the degradation for Asn21 and Asp60 and increases the degradation for Asn52, Asp53, Asn71, and Asp75 (Paper III).

The effect of polysorbate 80 on the thermal stability and structure of GA-Z

Polysorbate 80 at the investigated concentrations does not affect either the thermal stability or structure of GA-Z. Moreover, adding polysorbate 80 does not affect the solvent properties of the stability samples in a way that could potentially affect the chemical stability (18, 52, 66) to any significant extent. The inability of polysorbate 80 to influence the chemical stability of GA-Z is most likely due to its inability to change the solvent properties, thermal stability, and structure of GA-Z (Paper III).

The effect of DDM on the thermal stability of GA-Z

DDM lowers the thermal stability of GA-Z significantly. Figure 13 shows how DDM changes the DSC thermograms of GA-Z when adding 1.1, 5.5, and 10.9 mM DDM. When increasing the DDM concentration, the second peak corresponding to the unfolding transition of the albumin-binding domain gradually disappears. The disappearance may indicate that the albumin-binding domain loses its structure (Paper III).

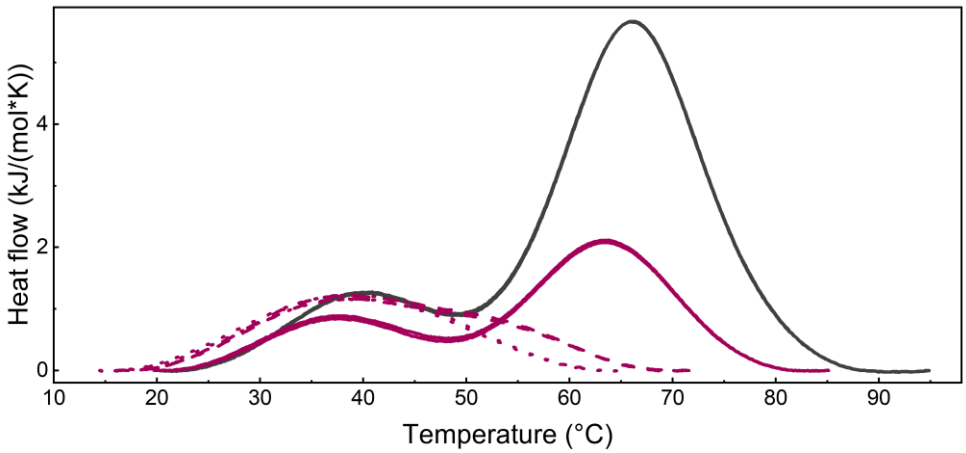

Figure 13. The DSC thermograms of GA-Z in 0-10.9 mM DDM. The samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0) and 0 (—) 1.1 (—), 5.5 (---), and 10.9 (···) mM DDM. The measurements are performed in duplicate and are included in the figure.

DDM-induced structural changes of GA-Z

DDM-induced structural changes were investigated using titration fluorescence and two-dimensional NMR spectroscopy at concentrations ranging from 0-15.3 mM (Paper III).

DDM-induced structural changes of the aromatic residues in GA-Z

Titration fluorescence spectroscopy was used to monitor the structural changes of the aromatic residues in GA-Z. GA-Z contains two tryptophane, three tyrosine, and two phenylalanine residues. Tyrosine and tryptophan contribute to the spectral profile due to the chosen excitation wavelength of 280 nm, with tryptophan dominating the emission spectra because of its high extinction coefficient (88). The emission spectra of GA-Z are recorded at DDM concentrations ranging from 0 to

15.3 mM at temperatures of 20 and 37 °C. Figure 14 shows that the addition of DDM significantly alters the environments of the fluorophores. In the absence of DDM, the emission peak maximum is 340 nm at 20 °C. A large blue shift occurs upon adding 3.8-4.2 mM DDM, with the emission peak maximum shifting from 338 to 333 nm. At 37 °C and 0 mM DDM, the emission peak maximum is lower, 338 nm, compared to at 20 °C. A significant blue shift is observed, with the emission peak maximum decreasing from 337 to 333 nm, and the blue shift is observed at lower DDM concentrations, between 1.2-1.5 mM DDM, compared to at 20 °C. Additionally, a second significant blue shift occurs, lowering the emission peak maximum from 332 to 328 nm at DDM concentrations between 11.0-12.9 mM. The DDM-induced blue shift indicates that the environments of the aromatic residues are becoming more hydrophobic (89).

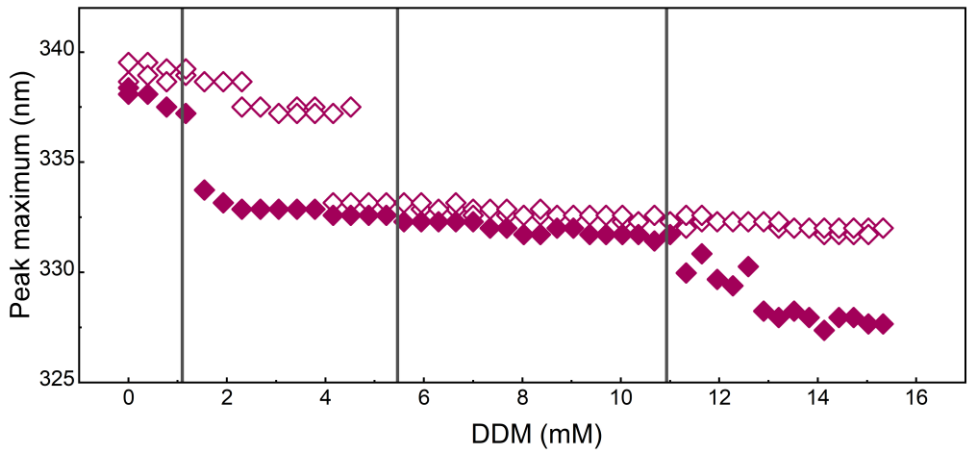

Figure 14. The emission peak maximums of the tryptophans and tyrosines in GA-Z at 0-15.3 mM DDM. The emission peak maximums are determined at 20 (◇) and 37 °C (◆). All samples contain 9 mg/mL GA-Z in PBS buffer (25 mM, 125 mM NaCl, pH 7.0). The black lines in the figure represent the concentrations of DDM in the stability samples. The measurements are performed in duplicate and are included in the figure.

DDM-induced structural changes observed with two-dimensional NMR spectroscopy

Two-dimensional NMR spectroscopy shows that adding 0-10.9 mM DDM changes the chemical shifts of some residues in the z-domain and in the albumin-binding domain. The chemical shift changes show that the chemical environments of the residues have changed and indicate that the structures of the residues in the two domains are altered. Moreover, the NMR data show that the albumin-binding domain does not lose its structure, as indicated by the gradual disappearance of the transition peak of the albumin-binding domain (Figure 13).

Interactions between DDM and GA-Z

As polysorbate 80 affects neither the chemical stability, the thermal stability, nor the structure of GA-Z, it is unlikely that polysorbate 80 interacts with GA-Z. In contrast, the changes in the chemical stability, the thermal stability, and in the structure of GA-Z that are induced by DDM, strongly indicate that DDM interacts with GA-Z. Both the head-group and the tail of DDM may bind to GA-Z. Since no interaction between polysorbate 80 and GA-Z is observed, hydrophobicity is probably not the primary driving force behind the interactions between GA-Z and DDM. Rather, the interaction likely originates in the maltose head-group of DDM. Sugars are known to bind to certain proteins (90, 91), and they typically interact with tryptophan, asparagine, and aspartic acid residues. Therefore, the DDM surfactants may associate with regions of GA-Z in which these residues are located. Furthermore, as previously mentioned, the observed blue shift in the emission peak maximums suggests that the tryptophan residue(s) in GA-Z have become more hydrophobic, possibly due to the DDM association (87).

The formation of a protein-micelle complex

Figure 15 shows how DDM changes the chemical stability and chemical environment of GA-Z. A change in the chemical environment indicates that the structure of the residue or its surroundings changes. The figure was created by combining the chemical stability data and chemical shift changes obtained from NMR spectra. Small changes in structure and stability are colored from green to yellow, and larger changes are colored from orange to red. In the z-domain, an orange/red area is observed, including the loop between α-helix 1 and 2, α-helix 3, and Ile16 positioned in the vicinity of α-helix 3. The orange/red area in the z-domain is close to the linker. In the linker, Asp60 is chemically stabilized by DDM. It is, therefore, likely that the linker is part of the orange/red area in the z-domain. An orange/red area is also observed in the albumin-binding domain, including the residues Asn71, Asp75, Lys84, and Ile103. The fluorescence data show that some or all of the tryptophan and tyrosine residues in GA-Z change their chemical environments as the concentration of DDM increases. In the z-domain, Trp27, situated in α-helix 2, is located adjacent to the observed orange/red area. Tyr83, in the albumin-binding domain, is positioned close to Lys84 and Ile103. No other tryptophan or tyrosine residues are located close to areas that change when DDM is added. The observed changes in the emission spectra are, therefore, probably caused by DDM-induced changes in the environments of Trp27 and/or Tyr83 (Figure 14).

Figure 15 shows that most of the observed DDM-indued changes occur on the same side of GA-Z, indicating that DDM primarily interacts with this side. One possible

explanation is that a DDM micelle interacts with GA-Z, leading to the formation of a complex between the protein and the micelle. The location of the observed changes in the protein may suggest that this complex is a decorated micelle, with the protein oriented such that one side primarily contacts the palisade layer of the DDM micelle. When adding DDM, the transition peak in the DSC thermograms corresponding to the albumin-binding peak gradually disappears (Figure 4). This disappearance may indicate that the domain has lost its structure. However, the NMR spectra do not show a complete loss of secondary or tertiary structure in the albumin-binding domain. The formation of a decorated micelle may reduce the conformational flexibility of GA-Z, which could explain the disappearance of Tm_2.

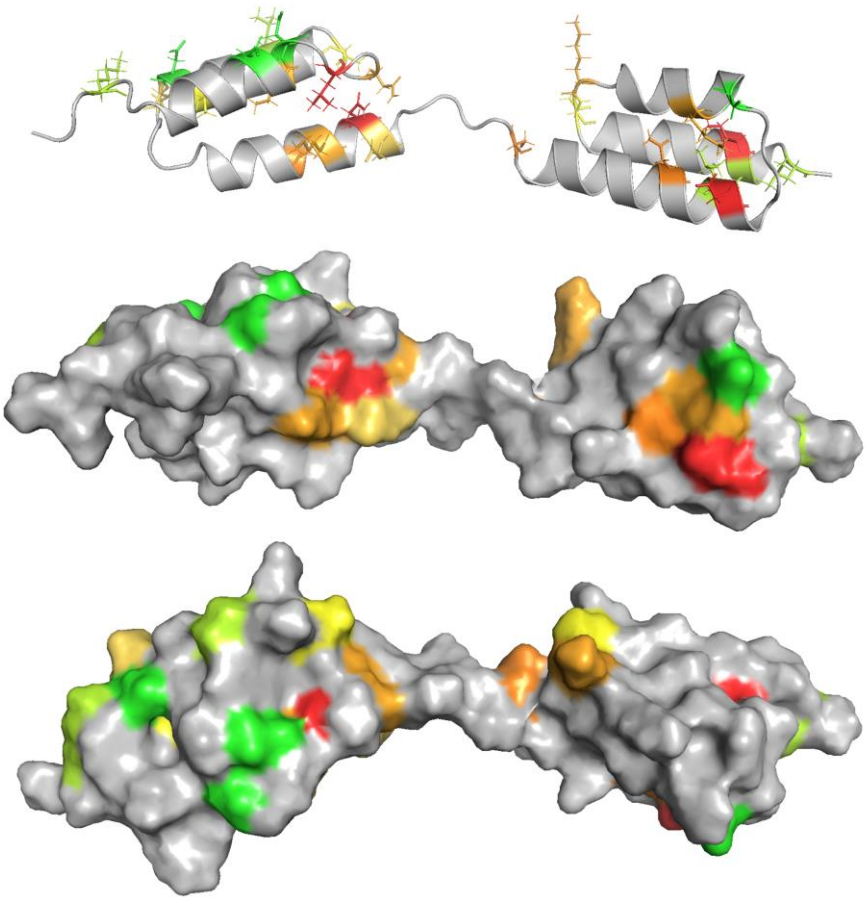

Figure 15. The areas where DDM-induced changes in the chemical stability or chemical environment. Residues that have changed their chemical stability or chemical shift in the presence of DDM are colored from orange to red. Unchanged residues are colored from green to yellow.

The DSC thermograms and emission peak maximums show that the structure of GA-Z changes in a stepwise manner with increasing concentrations of DDM (Figures 13 and 14). It is hypothesized that the following occurs at 37 °C: Between 0-1.2 mM DDM, unimers associate with GA-Z via their maltose head-groups, generating a blue shift in the emission peak maximum. Between 1.2 and 1.5 mM DDM, a larger blue shift is observed, likely caused by a significant conformational change of Trp27 induced by DDM interactions. Between 1.5-11.0 mM DDM, more DDM unimers bind to GA-Z, and the increasing number of DDM molecules interacting with GA-Z leads to the formation of a decorated micelle that includes both domains.

The effect of DDM on the chemical stability of GA-Z

As previously stated, adding DDM both increases and decreases the chemical stability of the residues in GA-Z. The residues that show increased degradation have likely obtained structures that have increased the propensity for deamidation and isomerization. It is structural changes that have increased the conformational flexibility of the peptide backbone of the residues or reduced the Cγ-N distances. Asn52 and Asp53 positioned in a-helix 3, in the z-domain, and Asn71 and Asp75 positioned in α-helix 1 in the albumin-binding domain degrade more when DDM is added. These residues could have lost their α-helix structures in the decorated micelle, which would significantly increase the conformational flexibility of their peptide backbone. A DDM-induced structural change of the residues could also have decreased their Cγ-N distances. On the other hand, the residues Asn21 and Asp60 are positioned in loops and are stabilized by DDM. The formation of a decorated micelle may reduce the conformational flexibility of the loops where these residues are situated and, therefore, lower their degradation.

A developed *in vitro* method used to investigate the stability and behavior of therapeutic proteins in plasma

A novel *in vitro* methodology that investigates and pre-screens the behavior of therapeutic proteins in plasma prior to *in vivo* analyses was developed. The method combines separation and characterization using AF4 with detection using LC-MS (Paper IV). Plasma is a complex matrix containing proteins, lipids, sugars, regulatory substances, nutrients, waste products, and salts (4, 78, 79). There can be more than 10 000 different proteins in plasma (92, 93). However, the most common plasma proteins are albumin, immunoglobulins, and fibrinogen, which together constitute over 90 wt% of the protein content (79). The high complexity of plasma puts high demands on the methods used to analyze it.

AF4 is a separation technique that separates analytes according to size, and analytes of small hydrodynamic sizes elute first (94, 95). The separation obtained with AF4 can have a large size range, which is necessary when separating plasma. After the separation with AF4, the eluent can be connected to multiple detectors measuring, for example, UV, multi-angle light-scattering, and differential refractive index, which can generate highly useful information about size, concentration, molecular weight, etc. An advantage of using AF4 for the separation and characterization of plasma is that the analysis can be designed to mimic the physiological conditions *in vivo*, i.e., it can be designed to have physiological salinity and pH. The addition of surfactants and organic solvents is not required (96), and the stress on the plasma samples is relatively low, as AF4 does not have a stationary phase, which generates a small internal surface area and low shear forces (97, 98). Due to the large number of different plasma proteins, there will most often be an overlap in the hydrodynamic sizes of the analyzed therapeutic protein and the plasma proteins, and co-elution is inevitable. Hence, another method is required to selectively identify and quantify the therapeutic protein.

LC-MS is a separation and detection technique with high selectivity, specificity, precision, and accuracy. LC-MS can be used to detect, identify, and quantify multiple proteins at once. LC-MS is, therefore, suitable for the complex analysis of plasma (4, 75, 76).

The binding of GA-Z to human serum albumin in plasma

The behavior of GA-Z in plasma is examined using the novel *in vitro* methodology. The results are shown in Figure 16. GA-Z was incubated in plasma for 30 min at 20 °C. GA-Z in plasma was then analyzed and separated using AF4-UV. The fractogram of the separation is shown by the green dashed line. A fractogram of the separation of GA-Z is shown by the orange dashed line, and a fractogram of the separation of plasma is shown by the black line. The elution peak of GA-Z has a maximum at the elution time of 3.1 min, corresponding to a hydrodynamic radius of approximately 2.9 nm. The first peak in the plasma fractogram is the elution of HSA. After incubating GA-Z in plasma, the peak corresponding to HSA is broadened to later elution times, corresponding to larger sizes. The formed complex is likely GA-Z bound HSA. During the separation of GA-Z in plasma, the eluent was collected in 1-minute fractions. The fractions were then trypsin-digested and analyzed with LC-MS. The relative amounts of GA-Z in the collected AF4 fractions are shown by the blue squares. 80% of GA-Z elutes between 4-5 minutes, where the broadening of the HSA peak is seen. The result confirms that the formed complex is GA-Z bound HSA. The molar ratio between GA-Z and HSA is approximately 1:3. In the fractogram of the separation of GA-Z in plasma, there is no peak at the elution times corresponding to unbound GA-Z, 2-4 min. The LC-MS data show that there are no GA-Z molecules in the fraction collected between 2-3 min. The result indicates that all GA-Z molecules bind to HSA. The LC-MS analysis also shows that small quantities of GA-Z proteins elute between 6-7 min. The later elution time may indicate that GA-Z binds to oligomers of HSA or that GA-Z binds to other plasma proteins of larger size. The later elution time may also correspond to the formation of oligomers of GA-Z or aggregated GA-Z.

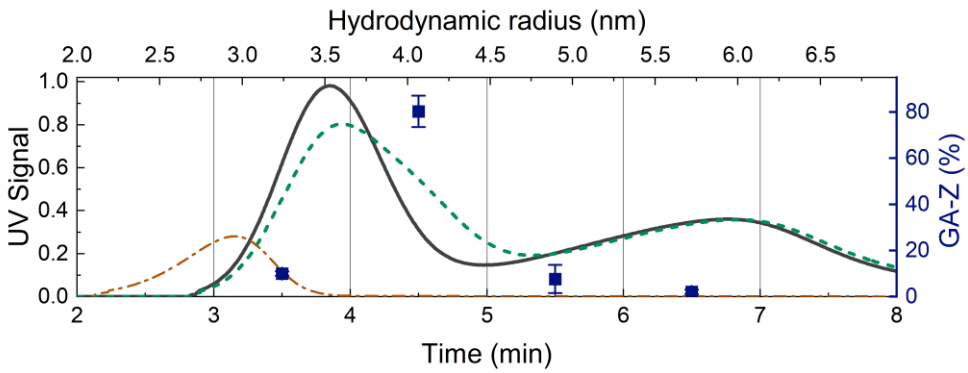

Figure 16. AF4-UV fractogram and the relative content of GA-Z. AF4-UV fractogram of samples with plasma (—, ~70 µg plasma proteins), GA-Z (---, 5 µg), and GA-Z incubated in plasma (---, 0.90 µg GA-Z + ~70 µg plasma proteins) for 30 min at 20 °C, in PBS buffer at pH 7.4.

The detection of aggregated trastuzumab in plasma

Aggregated trastuzumab in plasma was investigated using the novel *in vitro* methodology to determine whether this method can be used to detect and quantify aggregated. The results are shown in Figure 17. As for GA-Z, trastuzumab in plasma was incubated for 30 minutes at 20 °C. The aggregates of trastuzumab had been induced prior to the incubation in plasma. Aggregated trastuzumab in plasma was then analyzed and separated using AF4-UV, and 1-minute fractions were collected, trypsin-digested, and analyzed with LC-MS. The fractogram of the separation of aggregated trastuzumab in plasma is presented by the green dashed line. A fractogram of the separation of aggregated trastuzumab is shown by the orange dashed line, a fractogram of the separation of native trastuzumab is shown by the purple dashed line, and a fractogram of the separation of plasma is shown by the black line. The plasma peak, having a maximum at the elution time of 8.9 min corresponding to a hydrodynamic radius of approximately 5.8 nm, are plasma IgG molecules. As expected, the native trastuzumab elutes at the same time as the plasma IgG molecules, being an IgG antibody. The fractogram of aggregated trastuzumab has two peaks. The first peak corresponds to native trastuzumab, while the second peak corresponds to aggregated trastuzumab. In the fractogram of aggregated trastuzumab in plasma, there is a small increase in the IgG peak, showing the co-elution of native trastuzumab and plasma IgG. There are no clear signs of the aggregates of trastuzumab in the fractogram. However, the LC-MS analysis shows that trastuzumab elutes in the fractions collected between 10-14 min, with a second maximum between 12 and 13 minutes, which is the aggregated trastuzumab. In this study, the aggregates were induced before incubation in plasma. However, the result proves that the method can be used to detect aggregates and would, therefore, show whether aggregates are produced during the incubation of therapeutic proteins in plasma.

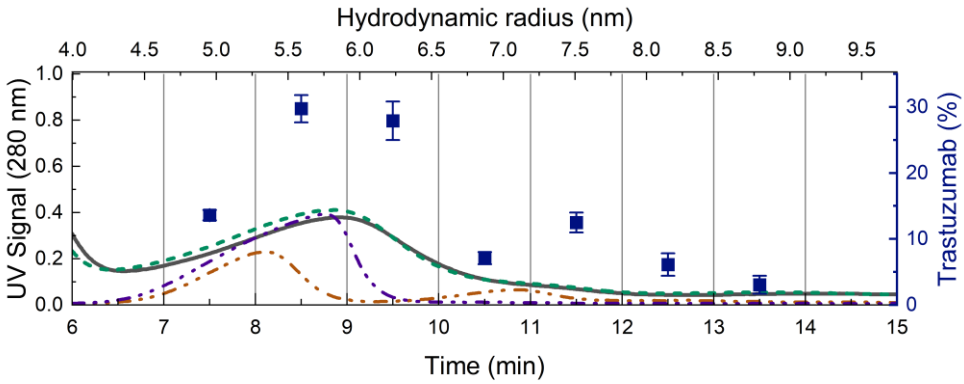

Figure 17. AF4-UV fractogram and the relative content of trastuzumab. AF4-UV fractogram of samples with plasma (—, ~70 µg plasma proteins), aggregated trastuzumab (---, 5 µg), trastuzumab (---5 µg), and aggregated trastuzumab incubated in plasma (---, 0.82 µg aggregated trastuzumab + ~70 µg plasma proteins) for 30 min at 20 °C, in PBS buffer at pH 7.4.

Conclusions

GA-Z degrades by deamidation, isomerization, and hydrolysis. A structural analysis of deamidated and isomerized residues of GA-Z shows that degradation is connected to structural motifs, and that residues with high conformational flexibility, a sequence motif, and/or shorter Cγ-N distances have lower chemical stability. The stability study shows that most of the degradation occurs in the z-domain. The denaturation temperatures of GA-Z and a structural analysis performed with NMR spectroscopy show that the z-domain is partly unfolded during storage at 37 °C. The high amount of degradation in the z-domain most likely results from the z-domain being in an unfolded state, increasing the conformational flexibility of the peptide backbone.

When introducing glycerol, fructose, sucrose, or melezitose, GA-Z is both stabilized and destabilized against deamidation and isomerization. However, the stabilizing effect is more dominant than the destabilizing effect. Asparagine residues are stabilized at all positions. Aspartic acids are destabilized in unstructured regions at polyol concentrations ranging from 0-50% v/v. The destabilization is attributed to a change in the solvent properties caused by the polyols.

The polyols increase the denaturation temperatures of GA-Z, thereby shifting the z-domain toward a folded state, which is likely the dominant factor contributing to the chemically stabilizing effect of polyols on GA-Z. However, it is concluded that stabilizing the folded state is not the only stabilizing factor. The polyols also induce a small structural change of the albumin-binding domain toward a more ordered state. This shift toward the more ordered state most likely stabilizes the residues against chemical degradation. A similar structural change likely occurs in the z-domain when 30-90% v/v glycerol is added, and this change increases the chemical stability of the residues in the domain. Stabilization of GA-Z at higher glycerol concentrations may also be correlated with a decrease in water activity. Moreover, it has been shown that polyols most likely increase the chemical stability of GA-Z through the preferential exclusion of polyols from its surface.

Polysorbate 80 does not affect either the stability or structure of GA-Z. In contrast, DDM affects the chemical stability, reduces thermal stability, and alters the structure of GA-Z. The observed changes are believed to result from DDM interacting with the GA-Z, driven by the maltose head-group of DDM. It is

hypothesized that these interactions lead to the formation of a protein-micelle complex, which modifies the stability of GA-Z.

A novel *in vitro* methodology has been developed that provides insight into the behavior of therapeutic proteins *in vivo*. The method involves a combination of AF4 and LC-MS. It is shown that after incubation of proteins in plasma, this method can be used to investigate and quantify serum albumin binding, analyze changes in monoclonal antibody size, and identify and quantify monoclonal antibody aggregates. Thus, this methodological approach opens many possibilities for further studies on the behavior and stability of therapeutic proteins.

Future studies

This thesis does not provide any knowledge about how glycerol and sugars change the molecular structure of the degradation residues of GA-Z. The knowledge could give a deeper understanding of how glycerol and sugars affect deamidation and isomerization. NMR experiments, such as NOESY, could provide molecular structural information on the degraded residues of GA-Z and show how glycerol and sucrose change their structure. The intensities of the peaks in a NOESY spectra can be used to calculate the distances between correlating atoms and, therefore, be used to follow structural changes. Simulations could also provide structural information with atomic resolution and could be used to follow glycerol-induced structural changes over time.

In this thesis, it is concluded that DDM interacts with GA-Z, and it is hypothesized that DDM and GA-Z form a protein-micelle complex. It would be very interesting to examine whether and how this complex is formed and, in detail, determine its structure. For the analysis, structural characterization methods, such as NMR spectroscopy and SANS, could be used. It would, as for glycerol and sugars, also be useful to analyze the system with simulations.

Additionally, the novel *in vitro* methodology combining AF4 and LC-MS could be used to further investigate the functionality and stability of GA-Z in plasma. It could be used to examine if deamidated GA-Z binds to Complement C5 and albumin, if unfolded GA-Z binds to Complement C5 and albumin, if the rate of deamidation changes under physiological conditions in plasma, and if deamidation of GA-Z leads to unwanted interactions and aggregation in plasma.

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