Affinity Determination of Protein A Domains to IgG subclasses by Surface Plasmon Resonance

Sofia Nohldén

February 22, 2008

LiTH-IFM-EX-08/1921-SE
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Supervisors
Tomas Björkman
Gustav Rodrigo
GE Healthcare, Uppsala

Examiner
Carl-Fredrik Mandenius
Department of Physics, Chemistry and Biology
Linköping University
ABSTRACT

A capture step with protein A is the most common purification step in the downstream purification process of monoclonal antibodies. It is therefore of great importance to increase the knowledge of the interactions involved in this purification technique. The purpose of this master thesis project was to determine the affinity of protein A domains to IgG subclasses by surface plasmon resonance (SPR).

Besides the five homologous IgG-binding protein A domains (E, D, A, B, and C) an engineered domain, similar to domain B and used in the protein A media MabSelect Sure™ (GE Healthcare) was included in the study. The domains were expressed in *E. coli*, affinity purified and immobilized onto sensor chip surfaces by amine coupling. The antibodies used in the interaction analyses were of the human IgG subclasses 1, 2, 3, and 4. Affinity determination was performed by kinetic analyses with the SPR-biosensor Biacore™ 2000.

All human IgG subclasses except IgG₃ were shown to bind to all protein A domains including the monomer of the SuRe ligand. The equilibrium constants, $K_D$-values, obtained were all in the low nanomolar range. For IgG₁ and IgG₄, no significantly differences in the affinity to any of the protein A domains were found, except for domain E where there might be quality issues of the prepared domain. Furthermore, a detected quality issue with the commercial IgG₂ made it impossible to determine the $K_D$-values for this subclass with any reliability.
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD</td>
<td>Albumin binding domain</td>
</tr>
<tr>
<td>CV</td>
<td>Column volume</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethyl formamide</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>F_ab fragment</td>
<td>Fragment antigen binding</td>
</tr>
<tr>
<td>Fc</td>
<td>Flow cell</td>
</tr>
<tr>
<td>F_c fragment</td>
<td>Fragment crystallisable</td>
</tr>
<tr>
<td>HAc</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>IFC</td>
<td>Integrated micro-Fluidic Cartridge</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix Assisted Laser Desorption Ionization- Time Of Flight</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDEA</td>
<td>2-(2-pyridylthio)ethaneamine hydrochloride</td>
</tr>
<tr>
<td>RI</td>
<td>Refractive index</td>
</tr>
<tr>
<td>RU</td>
<td>Response unit</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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</table>
1 INTRODUCTION

Monoclonal antibodies constitute one of the largest product groups in the biopharma industry today. They are used as treatment of cancer diseases as well as immune and inflammatory disorders. The manufacturing processes for monoclonal antibodies typically include expression in recombinant mammalian cell cultures and subsequent purification steps by centrifugation, chromatography and filtration, together with virus clearance. The most common purification step is capture of the antibodies by protein A affinity chromatography. Due to the very high selectivity, purification with this technique results in a very high purity in one single step.

Protein A is a protein originating from *Staphylococcus aureus* that consists of five homologous domains. These are independently capable of binding IgG, the class of antibodies to which therapeutic antibodies belong. It is known that protein A bind specifically to the IgG subclasses IgG$_1$, IgG$_2$, and IgG$_4$, but lacks detectable binding to the subclass IgG$_3$. However, the affinity constants of the different IgG-binding domains of protein A to the different IgG subclasses have not earlier been studied. This information is of interest to increase the knowledge of these interactions, and also important in future product development of protein A chromatography media.

This master thesis project aimed to determinate these affinity constants by surface plasmon resonance. The individual protein A domains were produced, and the affinity constants were determined by interaction analyses using a Biacore instrument.

The project was performed at Antibody Processing, Processing & Applications, GE Healthcare AB, Uppsala, between September 2007 and February 2008.
2 BACKGROUND

2.1 ANTIBODIES

Antibodies, also called immunoglobulins, are proteins produced by the immune system in response to the presence of a foreign substance, an antigen. The specific binding of antibodies to antigens signals foreign invasion and triggers immune responses responsible for putting the invading pathogens out of action. [1]

Antibodies can be produced as either monoclonal antibodies (mAbs) or polyclonal antibodies (pAbs). Antibodies that are produced by clones of a single antibody producing cell, and therefore identical, are called mAbs. Antibodies produced by different cell lines, pAbs, constitute a heterogeneous mixture of antibodies with different antigen binding properties. [1]

2.1.1 STRUCTURE AND CLASSIFICATION

In human serum there are five different classes of antibodies, with different biological properties. Immunoglobulin G (IgG) is the antibody present in highest concentration. The other classes of antibodies are called IgA, IgM, IgD and IgE. All antibodies are composed of two different types of polypeptide chains, a light (L) chain, with a molecular weight (MW) of 25 kDa, and a heavy (H) chain with an MW of 50 kDa. The light chain exists in two different types, κ and λ, and is present in all the classes of antibodies. The type of the heavy chain, on the other hand, is what determines the class of an immunoglobulin. The heavy chain of IgG is called γ and the heavy chains of immunoglobulin IgA, IgM, IgD and IgE are called α, μ, δ and ε respectively. [1, 2]

In addition to the grouping of antibodies into different classes, IgG and IgA can be further divided into subclasses. There are, for example, four different subclasses of human IgG: IgG₁, IgG₂, IgG₃, and IgG₄. These subclasses are distinguished by having different heavy chains which are called γ₁, γ₂, γ₃, and γ₄ respectively. [2]

The basic structural unit of an immunoglobulin molecule can be seen in Figure 1. The molecule consists of two identical L chains (shown in green) and two identical H chains (shown in blue) in a conformation that resembles the letter Y. The two H chains are linked together with disulfide bonds, and in the same way each L chain is linked to one of the H chains. Both L chains and H chains have a variable sequence at their N-terminal ends and a constant sequence at their C-terminal ends. Chains of the same type (κ or λ for L chains and γ, α, μ, δ or ε for H chains) have constant regions that are more or less identical whereas the variable regions can differ significantly. [1, 2]
In both L chains and H chains there are repeated homologous sequences. Each of these segments contains one intrachain disulfide bond and is folded into a three-dimensional structure called immunoglobulin domains. As shown in Figure 1 each L chain comprises two such domains, one constant (C) and one variable (V). H chains contain in general four immunoglobulin domains each. Three of them are in the constant region (C\textsubscript{H1}, C\textsubscript{H2} and C\textsubscript{H3}) and one is in the variable region (V\textsubscript{H}). \cite{1, 2}

Treatment of antibodies with the proteolytic enzyme papain cleaves the molecule into three fragments (Figure 2) each with a MW of 50 kDa. Two of these fragments, the F\textsubscript{ab} fragments, are identical and contain the antigen-binding activity of the antibody. The F\textsubscript{ab} fragments correspond to the L chain linked to the domains V\textsubscript{L} and C\textsubscript{L1} of the H chain. The third fragment has been observed to crystallize readily and is therefore called the F\textsubscript{c} fragment (fragment crystallisable). This fragment corresponds to the domains C\textsubscript{H2} and C\textsubscript{H3} of the H chains. \cite{3}
2.1.2 **THERAPEUTIC ANTIBODIES**

Today monoclonal antibodies constitute the second largest biopharmaceutical product category after vaccines. Of all biopharmaceuticals in clinical trials, 20 % belong to this product group. [4] A majority of the about 20 monoclonal antibodies on the market today are used as treatment of cancer diseases. Other major target indications include immune and inflammatory disorders. [5, 6]

Through the development of monoclonal antibodies the target specificity in the treatment of diseases, such as those mentioned above, has become more viable. The goal of antibody-based therapies is to eliminate or neutralize the pathogenic infection or the disease target. There are three different ways in which therapeutic antibodies can work to achieve this. The first alternative is that the antibody blocks the action of a specific molecule, for example a growth factor or any other mediator. This is achieved by binding of the antibody to the factor itself or to the receptor of the factor. The second alternative is targeting where the antibody, engineered to carry a molecule able to eliminate the target molecule, is directed towards specific populations of cells, for example cancer cells. The third alternative is that the antibody functions as a signal molecule that can induce for instance cell division or apoptosis. [4]

Besides the therapeutic antibodies there are a number of fusion proteins with therapeutic use. [5] An example is the 150 kDa \( F_c \)-fusion protein Etanercept that is used for treatment of rheumatoid arthritis. The molecule consists of the \( F_c \) region of an antibody while the \( F_{ab} \) regions are replaced by two tumour necrosis factor (TNF) receptors. [7]

2.1.3 **PRODUCTION AND PURIFICATION OF ANTIBODIES**

Therapeutic antibodies have relative low potency and must therefore be given in high doses. Moreover, they are often used for chronic diseases, where the treatment is going on for a long period of time. Consequently, large amounts of purified product, 10-100 kg, must be produced each year to meet the market demand. The large quantities of product needed for each patient also makes monoclonal antibodies to the most expensive of all drugs. The annual cost per patient can reach $35,000. [8]

To meet the demands of antibody production it is desirable to have highly productive and consistent manufacturing processes. Monoclonal antibodies on the market today are to a great extent manufactured using similar processes, often divided into two major areas; the upstream cell cultivation and the downstream purification processes. The most popular expression system for monoclonal antibodies is recombinant mammalian cell culture. The cell lines most widely used for this purpose are Chinese hamster ovary (CHO) and murine myeloma (NS0). The downstream processes aim to eliminate impurities in the product. These impurities can be product-related, like dimers, aggregates, fragments and various isoforms of the product, or process-related, like host cell protein (HCP), nucleic acids (DNA, RNA), leached protein A from the capture step and cell culture medium components. [6] The downstream process starts with cell removal by either centrifugation or microfiltration. The next step is normally a capture step
by affinity chromatography (see section 2.4.1) with protein A as the ligand. To remove remaining impurities, additional chromatography steps like ion exchange chromatography and hydrophobic interaction chromatography (HIC) are often used. Besides the chromatography steps, intermediate unit operations of filtration and viral clearance are normally also included in the manufacturing process. [5]

2.2 PROTEIN A

Protein A is a protein of *Staphylococcus aureus* that is displayed on the surface of the bacterium [3]. This protein contains five nearly homologous domains named E, D, A, B, and C in order from the N-terminus [9]. Binding studies have shown that each of these domains has the ability to bind IgG [9, 10]. The primary binding site for protein A on the IgG molecule is in the Fc region, between the two domains C\textsubscript{H}2 and C\textsubscript{H}3. [3] However, staphylococcal protein A (SpA) does not bind to all four of the subclasses of human IgG. It has been shown that SpA binds strongly to the subclasses 1, 2 and 4 while it shows only weak interaction with IgG\textsubscript{3}. [11] In addition to the Fc interaction, it has been shown that each domain has an affinity to the F\textsubscript{ab} fragments of certain antibodies. [12]

**Protein A chromatography**

Protein A affinity chromatography (see section 2.4.1) is widely used as a capture step in purification of antibodies. Due to very high selectivity, this step can provide a very high enrichment factor. More than 98% of the impurities can be removed in one single step. [9] Consequently, by introducing this purification step early in the purification chain, the number of successive unit operations can be reduced. Among the advantages of protein A chromatography are also that the purification procedure is fast and easy to use. A disadvantage of the technique is that there is always a small degree of leakage of the ligand protein A. [13]

As a complement to existing protein A media GE Healthcare has developed MabSelect SuRe, a medium with the advantages of being resistant to alkali treatment and offering a low degree of leakage. The MabSelect SuRe ligand (see Figure 3) is a tetramer of a domain developed by protein engineering of domain B in protein A. [14]
Figure 3. Protein engineering of protein A yields an alkali-stabilized tetramer variant. MabSelect SuRe is a tetramer of an alkali-stabilized domain, developed by protein engineering of one of the IgG-binding domains of protein A, domain B.

2.3 PROTEIN ANALYSIS

2.3.1 MALDI-TOF
Matrix-assisted laser desorption ionization-time-of-flight spectrometry (MALDI-TOF) is a commonly used mass spectrometric method. Protein samples, embedded in an appropriate matrix are dried onto a metal or ceramic slide, and then ionized by application of a laser beam. The ions formed are accelerated in an electric field and fly through a flight tube towards a detector. The time it takes them to reach the detector is determined by their mass and charge, and the precise mass is determined by analysis of proteins with a single charge. [1, 2]

2.3.2 GEL ELECTROPHORESIS

SDS-PAGE
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common technique for separating proteins by size. Prior to analysis the proteins are treated with the negatively charged detergent sodium dodecyl sulfate, SDS, and are thereby unfolded into polypeptide chains. During electrophoresis, the SDS-protein complexes are applied to a cross-linked polyacrylamide gel. An electric current is applied which makes the negatively charged complexes move towards the anode. Since the effect of differences in shape and charge are eliminated by the SDS treatment, the proteins in the gel separate into discrete bands according only to size. Small proteins are able to move through the pores in the gel and thereby migrate faster than larger ones. [2, 15]
**Microchip gel electrophoresis**

Using instrumental platforms based on the lab-on-chip technology electrophoretic separation, as well as other laboratory tasks, can be performed on small chips with microfluidic interconnected channels etched into it. The technique enables performance of several sequential experimental steps together with automated data analysis in one process. Compared to conventional techniques this technology has several advantages such as minimal sample requirement, ease of use and rapid analyses. [16]

### 2.4 CHROMATOGRAPHY

Chromatography is a technique for separating molecules according to their different properties. Proteins are most often separated by column chromatography. A solution containing a mixture of proteins that are to be separated is then applied to a column containing a porous solid matrix. As the proteins migrate through the column they are retarded to different extents depending on their interactions with the matrix. Depending on the choice of matrix, proteins can be separated according to charge, hydrophobicity, size or their ability to bind to other molecules. [2]

#### 2.4.1 AFFINITY CHROMATOGRAPHY

In affinity chromatography, proteins are separated according to their ability to interact with other compounds. Specific and reversible interaction between a protein and a molecule coupled to the matrix, a ligand, enables purification of the protein. The target molecules, applied to the column after equilibration with binding buffer, bind to the ligand molecules whereas other molecules are washed away. The interaction between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, van der Waal’s forces or hydrogen bondings. To elute the target molecule in purified form, the conditions have to be changed so that this interaction is reversed. Depending on the type of interaction, this can be performed by changing the pH, ionic strength, polarity or by using a competitive ligand. [17]

#### 2.4.2 SIZE EXCLUSION CHROMATOGRAPHY

In size exclusion chromatography (SEC), also called gel filtration, molecules are separated according to their size. The chromatographic medium used in this technique is a gel with pores that have a carefully controlled range of sizes, comparable in size to the molecules to be separated. [18]

Relatively small proteins diffuse into the gel particles on their way through the column whereas larger proteins, prevented by their size from diffusing into the gel to the same degree, remain in the mobile phase. Consequently, the largest particles, which move outside the beads, leave the column first followed by the smaller molecules in the order of size. [18]
2.5 SURFACE PLASMON RESONANCE

Surface plasmon resonance (SPR), an optical phenomenon described in section 2.5.2, enables monitoring of biomolecular interactions in real time. Biacore instruments are biosensors based on this technique. The basic experimental principle is that one of the components in the interaction is immobilized on a sensor chip (see section 2.5.3) whereas the other component is flowed over the sensor chip surface in free solution. By definition, the immobilized component is called the ligand and the component in free solution is referred to as the analyte. As the interaction proceeds the concentration of the analyte in the surface layer changes and gives rise to an SPR response that can be followed in real time. [19]

2.5.1 INSTRUMENTATION

In a Biacore system there are three main components: the sensor chip, the optical system and a liquid handling system. In the optical system the SPR signal is generated and monitored. The liquid handling system consists of two pumps, for maintaining continuous flow and handling samples respectively, and an integrated micro-fluidic cartridge (IFC) for controlled transport of liquid to the sensor chip surface. [19] In Figure 4 the most important components of Biacore 2000 are presented and the general principles for interaction analyses are described.

![Figure 4. A general outline of the Biacore 2000 processing unit.](image)

As a sensor chip (1) is docked into the instrument, the IFC is pressed against the sensor chip and four separate detection flow cells (2) are formed, where the sensor chip surface constitutes one of the sides. To maintain a stable temperature, which is important for reliable SPR measurements, a system for temperature control covers the sensor chip and larger part of the IFC. Of the two pumps (3) in the system, the left one is responsible for maintaining continuous flow through the detector flow cell. Running buffer is flowed from the buffer compartment (4) through the system and is then transferred to the waste bottle. The right-hand pump is used for transferring, diluting and mixing of samples as well as for injection of samples. The samples are injected into the IFC from the vials placed in the sample racks (5) by the delivery arm with needle in the autosampler compartment (6). The formation and dissociation of biomolecular complexes causes SPR signals, created and detected in the detector unit (7). [20]
2.5.2 Theoretical Background

Surface plasmon resonance (SPR) is the optical phenomenon that Biacore instruments use to detect binding interactions between molecules. The SPR method is based on the dispersion of light at the interface between two media of different refractive index (RI). In Biacore experiments the media with the highest RI correspond to the gold coated glass slide of the sensor chip and the media with the lower RI constitutes of the aqueous buffer. At a certain angle of incident, sometimes referred to as the SPR angle, the reflected light is markedly reduced. This phenomenon, called surface plasmon resonance, is due to energy transfer from the light to the electrons in the metal surface. Some of the energy from the reflected light excites the electrons in the gold coating. This generates an oscillation of the electrons, called a plasmon. The electrical field of the plasmon is affected by molecules binding to the metal surface and this in turn affects the reflected light beam. Thus, binding of analyte molecules to the ligand leads to a shift of the RI in the solution close to the surface of the sensor chip. This shift of the RI affects the SPR angle and is thereby detected, see Figure 5. The SPR response is proportional to the mass of the material bound to the chip surface. The signal is expressed in response units, RU, plotted against time in a sensorgram. For most biomolecules, 1000 RU (1 kRU) is equivalent to a change in surface concentration of approximately 1 ng/mm$^2$. [2, 3, 19]

![Diagram of SPR phenomenon](image)

**Figure 5. Principles of the SPR phenomenon.**
SPR biosensors detect changes in the refractive index near the surface of the sensor chip.

a) At a specific angle, the SPR angle, a sharp dip is observed in the reflected light.

b) When analyte molecules bind to the sensor chip surface, the refractive index in the solution near the surface changes and consequently the SPR angle changes.
2.5.3 SENSOR CHIP

There are a number of different types of sensor chips available for use with Biacore systems. The surfaces of the sensor chips of the different types have somewhat different compositions and are therefore suitable for analyses of different types of interactions. [21]

The most versatile of the sensor chips available is sensor chip CM5. It is designed for analysis of interactions involving a wide range of biomolecules such as proteins, lipids, carbohydrates and nucleic acids. [21] The chip is a glass slide with a thin layer of gold on one side. To the gold film a surface matrix of non-crosslinked carboxymethylated dextran is covalently bound and to this matrix biomolecules can be immobilized. The chip is mounted on a plastic support frame that is protected by a plastic cassette. [19]

2.5.4 IMMobilization

Depending on the selection of sensor chip and the purpose of the experiments, different coupling chemistries for immobilization of the ligand to the sensor chip surface are available. [21]

The most generally applicable coupling chemistry is amine coupling, see Figure 6a. In this coupling method the carboxymethyl groups of the surface matrix is modified with a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N’-(dimethyl-aminopropyl)-carbodiimide (EDC). This introduces N-hydroxysuccinimide esters into the matrix which then can react spontaneously with amines and other nucleophilic groups on the ligand to form covalent bonds. After coupling, remaining reactive groups on the surface are deactivated with ethanolamine. The high ionic strength of this solution removes non-covalently bound material from the surface. Figure 6b shows a typical sensorgram from immobilization of any protein to a sensor chip surface using the amine coupling chemistry. [22]

Most macromolecules contain many groups which can participate in the amine coupling reaction. The diversity of potential coupling sites might be a drawback for some applications. If necessary to control the orientation of the ligand, thiol coupling can be a useful alternative. [22]
**2.6 Kinetic Measurements**

There are several applications for Biacore experiments. Among the main application areas are determination of kinetic constants and analyte concentrations as well as analyses of binding patterns and complex formation. [22] In this project, affinity has been determined by kinetic measurements.

The kinetics of an interaction, *i.e.* the complex formation and dissociation can be determined from the information in a sensogram. A typical sensogram from injecting a discrete pulse of analyte across a ligand surface, and thereafter a pulse of regeneration solution, can be seen in Figure 7. The sensogram from the analyte injection can be divided into three essential phases; association, equilibrium and dissociation. A subsequent regeneration of the surface is done in order to remove remaining analyte molecules and thereby prepare the surface for the next analysis cycle. Evaluation of the sensogram generates two kinetic rate constants: association rate constant ($k_a$) and dissociation rate constant ($k_d$). For a simple 1:1 interaction, the equilibrium constant, $K_D$, is the ratio of the kinetic rate constants, $k_d / k_a$. Consequently, a lower value of $K_D$ corresponds to a greater affinity. [21]

To obtain successful kinetic determinations it is recommended to use a low level of immobilized ligand, this in order to make sure that the interactions are not limited by mass transport processes. A low immobilization level corresponds to a low maximum analyte binding capacity, referred to as $R_{max}$. The analyte should be diluted into a concentration series of at least 5 to 6 concentrations covering a range of 100-fold or more. To correct for bulk response, kinetic analyses require a reference surface of any type. This surface can be left unmodified, activated and deactivated to reduce non-specific binding, or it can be immobilized with a ligand known to not bind the analyte. [21]
2.7 **AIM OF THE STUDY**

The purpose of this master thesis project was to determine the affinity of individual protein A domains to different IgG subclasses using surface plasmon resonance.

The individual domains of protein A were expressed by cultivation of *Escherichia coli* (*E. coli*), and the products were thereafter purified by affinity chromatography and characterized. Method development for analyses by surface plasmon resonance was performed in order to find a method suitable for determination of affinity constants. Protein A domains were immobilized onto sensor chips, and their affinity to different IgG subclasses were studied by kinetic analysis.

---

*Figure 7. A typical sensorgram from an analyte injection with subsequent injection of regeneration solution.*
3 MATERIALS AND METHODS

3.1 CHEMICALS

The chemicals used for production and purification of protein A domains as well as IgG fragments are listed in Table 1, the chemicals used in the Biacore experiments in Table 2, and the chemicals used for protein analysis in Table 3. Milli-Q® water was used to prepare all solutions.

Table 1. Chemicals used for production and purification of protein A domains and IgG fragments

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
<th>Product no</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>Acetic acid</td>
<td>Merck</td>
<td>1.00063.2511</td>
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<tr>
<td>Aspartic acid Na salt</td>
<td>Calbiochem</td>
<td>189005</td>
</tr>
<tr>
<td>Carbencillin disodium</td>
<td>Saveen Werner AB</td>
<td>C0109.005</td>
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<tr>
<td>D(+)−glucose</td>
<td>VWR</td>
<td>24371.366</td>
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<td>D-lactose</td>
<td>KEBO</td>
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<tr>
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<td>Merck</td>
<td>1.08418.0250</td>
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<td>Glycerol, 87% w/v</td>
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<td>HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid)</td>
<td>Sigma</td>
<td>H4034</td>
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<tr>
<td>Immobilized papain</td>
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<td>MgSO₄ × 7 H₂O</td>
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</tr>
<tr>
<td>NaCl</td>
<td>Merck</td>
<td>1.06404.1000</td>
</tr>
<tr>
<td>NaH₂PO₄ × H₂O</td>
<td>Merck</td>
<td>1.06346.1000</td>
</tr>
<tr>
<td>NaOH</td>
<td>Merck</td>
<td>1.06469.1000</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Merck</td>
<td>1.01145.1000</td>
</tr>
<tr>
<td>PBS (20 mM phosphate, 0.15 M NaCl, pH 7.4)</td>
<td>Elsichrom AB</td>
<td>R113/1</td>
</tr>
<tr>
<td>Peptone S</td>
<td>Acumedia</td>
<td>7180 A</td>
</tr>
<tr>
<td>Tryptone</td>
<td>BD</td>
<td>211705</td>
</tr>
<tr>
<td>Tween® 20</td>
<td>USB</td>
<td>20605</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Acumedia</td>
<td>7184A</td>
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</table>
Table 2. Chemicals used in Biacore experiments

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
<th>Product no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
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<td>1.00063.2511</td>
</tr>
<tr>
<td>Boric acid</td>
<td>Merck</td>
<td>1.00165.1000</td>
</tr>
<tr>
<td>DTE (Ditioerythriol)</td>
<td>GE Healthcare, internal product</td>
<td>30073100</td>
</tr>
<tr>
<td>EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)</td>
<td>GE Healthcare</td>
<td>Part of Amine Coupling Kit, BR-1000-50</td>
</tr>
<tr>
<td>Ethanolamine-HCl, 1.0 M pH 8.5</td>
<td>GE Healthcare</td>
<td>Part of Amine Coupling Kit, BR-1000-50</td>
</tr>
<tr>
<td>Formic acid</td>
<td>Merck</td>
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<td>glycine-HCl, 10 mM, pH 1.5</td>
<td>GE Healthcare</td>
<td>Part of Regeneration Scouting Kit, BR-1005-56</td>
</tr>
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<td>glycine-HCl, 10 mM, pH 2.0</td>
<td>GE Healthcare</td>
<td>Part of Regeneration Scouting Kit, BR-1005-56</td>
</tr>
<tr>
<td>glycine-HCl, 10 mM, pH 2.5</td>
<td>GE Healthcare</td>
<td>Part of Regeneration Scouting Kit, BR-1005-56</td>
</tr>
<tr>
<td>glycine-HCl, 10 mM, pH 3.0</td>
<td>GE Healthcare</td>
<td>Part of Regeneration Scouting Kit, BR-1005-56</td>
</tr>
<tr>
<td>HBS-EP running buffer</td>
<td>GE Healthcare</td>
<td>BR-1001-88</td>
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<td>Hydrochloric acid</td>
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<td>NaCl</td>
<td>VWR</td>
<td>27810.295</td>
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<tr>
<td>NHS (N-hydroxysuccinimide)</td>
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<td>Part of Amine Coupling Kit, BR-1000-50</td>
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<tr>
<td>PDEA Thiol coupling reagent</td>
<td>GE Healthcare</td>
<td>BR-1000-58</td>
</tr>
<tr>
<td>Sodium formate</td>
<td>Merck</td>
<td>3634162</td>
</tr>
<tr>
<td>Tris-(hydroxymethyl)-aminomethan</td>
<td>Merck</td>
<td>1.08382.0500</td>
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<tr>
<td>Tween® 20</td>
<td>USB</td>
<td>20605</td>
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Table 3. Chemicals used for protein analysis.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
<th>Product no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitril</td>
<td>Merck</td>
<td>1.00030.2500</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Serva</td>
<td>28625</td>
</tr>
<tr>
<td>CyDye DIGE Fluor, Cy5 minimal dye</td>
<td>GE Healthcare</td>
<td>RPK0275</td>
</tr>
<tr>
<td>N,N-dimethyl formamide (DMF)</td>
<td>USB</td>
<td>14682</td>
</tr>
<tr>
<td>Sinapinic acid</td>
<td>Laser BioLabs</td>
<td>M104</td>
</tr>
<tr>
<td>Trifluoric acid</td>
<td>Merck</td>
<td>8.08260.0100</td>
</tr>
</tbody>
</table>
3.2 **ANTIBODIES**

Two different antibodies of the IgG₁, IgG₃, and IgG₄ subclasses respectively, along with one IgG₂ antibody, were used in the interaction analyses (Table 4). The antibody of IgG₂ and one of the antibodies of IgG₃ were ordered from Nordic Biosite AB. The antibodies from GE Healthcare were all produced and purified according to the general procedures described in section 2.1.3. In addition to the IgG subclasses, the fusion protein Etanercept was included in the study.

Table 4. Antibodies and Fc-fusion protein used in interaction analyses

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Denotation</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG₁</td>
<td>IgG₁a</td>
<td>GE Healthcare, internal product</td>
</tr>
<tr>
<td></td>
<td>IgG₁b</td>
<td>GE Healthcare, internal product</td>
</tr>
<tr>
<td>IgG₂</td>
<td>IgG₂a</td>
<td>Nordic Biosite AB, cat # A50184H</td>
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<tr>
<td></td>
<td>IgG₂b</td>
<td>GE Healthcare, internal product</td>
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<tr>
<td>IgG₃</td>
<td>IgG₃a</td>
<td>Nordic Biosite AB, cat # A50186H</td>
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<tr>
<td></td>
<td>IgG₃b</td>
<td>GE Healthcare, internal product</td>
</tr>
<tr>
<td>IgG₄</td>
<td>IgG₄a</td>
<td>GE Healthcare, internal product</td>
</tr>
<tr>
<td></td>
<td>IgG₄b</td>
<td>GE Healthcare, internal product</td>
</tr>
<tr>
<td>Fc-fusion protein</td>
<td>Etanercept</td>
<td>Amgen</td>
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</tbody>
</table>

3.3 **MATERIAL**

The materials used in the experiments are listed in Table 5.

Table 5. Materials used in the experiments.

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
<th>Product no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent Protein 230 Kit</td>
<td>Agilent</td>
<td>5067-1518</td>
</tr>
<tr>
<td>Agilent Protein 80 Kit</td>
<td>Agilent</td>
<td>5067-1515</td>
</tr>
<tr>
<td>Amicon Ultra–4 Ultracel-10k centrifuge filter</td>
<td>Millipore</td>
<td>UFC801024</td>
</tr>
<tr>
<td>HiLoad™ 16/60 Superdex™ 200 prep grade column</td>
<td>GE Healthcare</td>
<td>17-1069-01</td>
</tr>
<tr>
<td>HiPrep™ 26/10 Desalting</td>
<td>GE Healthcare</td>
<td>17-5087-01</td>
</tr>
<tr>
<td>IgG Sepharose™ 6 Fast Flow</td>
<td>GE Healthcare</td>
<td>17-0969-01</td>
</tr>
<tr>
<td>illustra NAP™-10</td>
<td>GE Healthcare</td>
<td>17-0854-01</td>
</tr>
<tr>
<td>illustra NAP™-5</td>
<td>GE Healthcare</td>
<td>17-0853-01</td>
</tr>
<tr>
<td>Immobilized papain</td>
<td>Pierce</td>
<td>20341-P</td>
</tr>
<tr>
<td>MabSelect SuRe™</td>
<td>GE Healthcare</td>
<td>17-5438-03</td>
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<tr>
<td>PhastGel™ 8-25</td>
<td>GE Healthcare</td>
<td>17-0542-01</td>
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<tr>
<td>PhastGel™ strips SDS</td>
<td>GE Healthcare</td>
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</tr>
<tr>
<td>Protein Chips</td>
<td>Agilent</td>
<td>5067-1523</td>
</tr>
<tr>
<td>Sensor chip CM5 (research grade)</td>
<td>GE Healthcare</td>
<td>BR-1000-14</td>
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<tr>
<td>Superdex™ 200 5/150 GL column</td>
<td>GE Healthcare</td>
<td>28-9065-61</td>
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<tr>
<td>Tricorn™ 5/100 column</td>
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<tr>
<td>XK 16/20 column</td>
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<td>18-8773-01</td>
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</table>
3.4 Instrumentation

The instrumentation used in the experiments is listed in Table 6.

Table 6. Instrumentation used in the experiments.

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Agilent 2100 Bioanalyzer</td>
<td>Agilent Technologies</td>
<td>G2938C</td>
</tr>
<tr>
<td>Biacore™ 2000</td>
<td>GE Healthcare</td>
<td>BR-1002-25</td>
</tr>
<tr>
<td>Novaspec® II spectrophotometer</td>
<td>Amersham Pharmacia Biotech</td>
<td>-</td>
</tr>
<tr>
<td>PhastSystem™</td>
<td>Pharmacia</td>
<td>18-1018-24</td>
</tr>
<tr>
<td>Typhoon™ 9410</td>
<td>GE Healthcare</td>
<td>63-0055-80</td>
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<tr>
<td>ÄKTA™ explorer 10</td>
<td>GE Healthcare</td>
<td>18-1300-00</td>
</tr>
<tr>
<td>ÄKTA™ explorer 100</td>
<td>GE Healthcare</td>
<td>18-1112-41</td>
</tr>
</tbody>
</table>

3.5 Expression of Protein A Domains

Vectors for the expression of the individual protein A domains, named E, D, A, B, and C, fused to the albumin binding domain (ABD) of staphylococcal protein G were kindly donated by Affibody AB. The protein domains were expressed as intracellular products in *Escherichia coli* (*E. coli*) BL21 DE(3). The growth of bacteria was controlled by measurements of the optical density (OD) at 600 nm in a 1 cm path length cuvette using a Novaspec® II spectrophotometer.

In the starting cultures prepared for expression of the protein A domains A, B, C and D the non-inducing medium MDG (Appendix A) was used. For expression of domain E the cultivation medium 2 YT (Appendix A) was used in the starting culture. Carbenicillin (100 mg/l) was added to shake flasks containing 25 ml medium. The medium 2 YT was also supplemented with glucose (5 g/l). After inoculation with cells from 250 µl of frozen glycerol stocks, the cultures were incubated at 37°C and 200 rpm for 6-7 h until OD at 600 nm reached a value between 1.5 and 2.

For production, shake flasks containing 500 ml of the auto inducer medium ZYP-5052 (Appendix A) were supplemented with carbenicillin (100 mg/l) and 0.1 ml of the antifoam agent Breox. The culture in each shake flask was inoculated with 5 ml of one of the starting cultures and grown at 37°C and 180 rpm overnight, resulting in OD values of about 7 to 11 at 600 nm.

The cell culture was dispensed into 250 ml centrifuge tubes and put on ice before centrifugation at 20 000×g for 15 minutes with the temperature set to 5°C. The supernatant was discarded, and the cell pellet resuspended in PBS before sonication at pulse mode (9 seconds + 9 seconds) at 33% amplitude for 3 minutes. Centrifugation of the sonicated suspension was performed for 15 minutes at 20 000×g with the temperature set to 5°C. The supernatant was placed in refrigerator until the following purification (see section 3.6).

The monomer of the SuRe ligand, also included in the study, was a GE Healthcare internal product expressed and purified in a similar way as the other protein A domains.
3.6 Purification of Protein A Domains

The E, D, A, B, and C domains, fused to ABD, were purified by IgG affinity chromatography. The purification was performed on IgG Sepharose™ 6 Fast Flow in an XK 16/20 column packed to a bed height of 14 cm. The work was carried out using an ÄKTA™explorer 100 system controlled by the software UNICORN™ 5.01.

Briefly, the gel was equilibrated with the loading buffer (25 mM sodium acetate, 200 mM sodium chloride, pH 5.8) for 0.25 column volumes (CVs). A CV of 28.1 ml and a flow rate of 5 ml/min (149 cm/h) resulted in a residence time of 5.6 minutes. After sample injection, the column was washed with six CVs of loading buffer followed by two CVs of 25 mM sodium acetate, pH 5.8. Elution of the bound material was carried out using 60 mM acetic acid, pH 3.0, and the eluate with an absorbance greater than 100 mAU was collected. The gel was then re-equilibrated with five CVs of loading buffer.

The absorbances of the eluates were measured spectrophotometrically at 276 nm. The concentrations of the samples, and the total amount of the products, were then calculated by use of the extinction coefficients of the domains. Before storage in refrigerator, the pH of the purified fusion proteins was adjusted with sodium hydroxide to a pH in the range 4-4.5 where the proteins are more stable. The fusion proteins were also sterilized by filtration (0.2 µm).

3.7 Preparation of IgG-Fragments

F<sub>ab</sub> and F<sub>c</sub> fragments of antibody IgG<sub>a</sub> were generated by enzymatic digestion with the proteolytic enzyme papain. The work was carried out using an ÄKTAexplorer 10 system controlled by the software UNICORN 5.01.

A Tricorn™ 5/100 column packed with immobilized papain was used. The bed height was 10.7 cm, corresponding to a column volume of approximately 2.1 ml. The antibody was buffer exchanged into digestion buffer (20 mM sodium phosphate, 10 mM ethylenediaminetetraacetic acid (EDTA), 20 mM cystein-HCl at pH 7.0) using the column HiPrep™ 26/10 Desalting. The resulting concentration of the antibody was 6.6 mg/ml. The column was equilibrated with digestion buffer, and a sample volume of 0.9 ml was applied. After a 90 minutes pause for incubation the sample was eluted. By use of a scouting procedure this process was repeated ten times. The eluates from all the runs in the scouting procedure were pooled.

To evaluate the enzymatic cleavage of the antibody IgG<sub>a</sub>, the eluates from the immobilized papain column were analyzed using size exclusion chromatography (SEC). The run was performed on an ÄKTAexplorer 10 system using phosphate buffered saline (PBS) pH 7.4 and a Superdex™ 200™ 5/150 GL column. A sample volume of 30 µl was applied to the column, and the flow rate was set to 300 µl/min.
3.8 Purification of IgG-fragments

To obtain pure Fc and Fab fragments, the eluates from the papain cleavage of the antibody IgG1 were purified using a Tricorn 5/100 column packed with MabSelect SuRe to a column volume of 1.9 ml. The system used was ÄKTAexplorer 10, controlled by the software UNICORN 5.01. Fc fragments and intact antibody molecules bind to the ligand molecules whereas the Fab fragments do not. The purification over the MabSelect SuRe column was performed in three cycles. The loading and equilibration buffer consisted of 25 mM sodium phosphate and 0.5 M sodium chloride at pH 7.3. After sample injection at a flow rate of 0.7 ml/min (214 cm/h), corresponding to a residence time of about 3 minutes, the column was washed with the loading buffer for five CVs. The elution was performed using 0.1 M sodium phosphate, pH 3.0 at a flow rate of 0.33 ml/min (101 cm/h). The eluted material with an absorbance greater than 100 mAU at 280 nm was collected. Before re-equilibration of the column, a cleaning-in-place procedure with 0.5 M sodium hydroxide for three CVs was performed.

The Fab fragments (flow through from MabSelect Sure) were concentrated using an Amicon filter. Of the Fc and Fab fragments pools, 2.2 and 5 ml respectively were purified on a HiLoad™ 16/60 Superdex 200 prep grade column. The running buffer consisted of 0.01 M HEPES, 0.15 M NaCl, and 3 mM EDTA, pH 7.4. Fractions of 1 ml each were collected during the elution procedures. After evaluation of the chromatograms fractions were pooled. Tween® 20 was added to the purified samples to a concentration of 0.05% after the runs were terminated.

3.9 Protein Analysis

3.9.1 Analysis of protein A domains

MALDI-TOF

The produced protein A domains and the monomer of the SuRe ligand was analyzed with Matrix Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF). The matrix, sinapinic acid, was dissolved to a concentration of 10 mg/ml in a solution of 40% acetonitril and 0.1% trifluoroacetic acid. The samples were diluted in Milli-Q water to a concentration of about 1 mg/ml. Reduction of the samples was performed by addition of 0.5 µl 1M β-mercaptoethanol to each sample of 5 µl followed by incubation at 37°C for 30 minutes. Prior to spotting of the protein sample onto a target plate, each sample was mixed with 5 µl of the matrix solution.

Microchip gel electrophoresis

The produced protein A domains were diluted into concentrations of about 0.6 mg/ml, and analyzed with microchip gel electrophoresis using Agilent 2100 Bioanalyzer and Agilent Protein 80 Kit. Sample preparation, using denaturing sample buffer, and loading of samples and ladder to the chip, were performed according to Agilent Protein 80 Assay Protocol.
3.9.2 Analysis of IgG and its fragments

Analysis of antibodies by microchip gel electrophoresis

The antibodies were analyzed using Agilent 2100 Bioanalyzer. Gel-dye mix and destaining solution were prepared using the materials and chemicals included in the Agilent Protein 230 Kit. The antibodies were diluted to a concentration of 1 mg/ml in Milli-Q water. Sample preparation, using non-reducing sample buffer, and loading of samples and ladder to the chip were performed according to the manufacturer’s protocol.

Analysis of prepared Fc and Fab fragments by SDS-PAGE

The Fc and Fab fragments were analyzed with SDS-PAGE using PhastSystem™ with PhastGel™ 8-25 and PhastGel™ Strips SDS. To 45 µl of each sample 5 µl of 200 mM Tris-HCl, pH 8.5 was added. 10 µl of each sample was then mixed with 1 µl Cy5, dissolved 2:15 in N,N-dimetyl formamide (DMF). After incubation in refrigerator for 30 minutes, the samples were mixed 1:1 with sample buffer (10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, 0.01% brome phenol blue, pH 8.0) and placed in a 95°C heating block for 3 minutes before application onto the gel. To detect the Cy5 labelled proteins, the gels were scanned with a Typhoon™ scanner using the 633 nm (red) laser for excitation.

3.10 Optimization of Biacore experiments

3.10.1 Instrumentation

For the interaction analyses performed the surface plasmon resonance (SPR) biosensor Biacore 2000 was used. The analyses were carried out at 25.0°C with a data collection rate of 1.0 Hz. Maintenance of the instrument was performed according to the supplier’s instructions. The software used for control of the instrument and for creation of new running methods was Biacore 2000 Control Software, version 3.2.1. For evaluation of the sensorgrams the software BIAevaluation, version 4.1 was used.

3.10.2 Running buffer

The running buffer HBS-EP was used in all interaction analyses performed. The buffer contains 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% v/v Surfactant P20. In the first experiments this buffer was used unmodified. After recommendations from Biacore AB [23] more Tween 20 (equivalent to Surfactant P20) was added to this buffer, in order to reduce non-specific protein interactions. A 10 % stock solution of Tween 20 was prepared by dilution of 1 ml Tween 20 in 9 ml Milli-Q water. To obtain a 0.05% Tween 20 buffer with 1 ml of this stock solution was then added to 200 ml of HBS-EP. The modified buffer was used in the experiments where low ligand levels where reached by immobilizing the ligand with a method with a shorter activation time.
3.10.3 Preparation of ligands

In the interaction analyses the produced protein A domains together with the monomer of the SuRe ligand were used as ligand molecules. The ligands were diluted in 10 mM acetic acid (HAc) giving a pH of 3.4. At this pH the protein A domains, which have a pI-value of about 5, are positively charged and thereby attracted to the negatively charged matrix of the sensor chip.

The ligands were diluted to concentrations low enough to enable immobilization to the desired levels. In the final experiments the ligand concentrations were in the range of about 4-7 µg/ml for the domains of protein A and about 20 µg/ml for the monomer of the SuRe ligand. To obtain the lowest ligand level possible when using the wizard “Aim for immobilized level” it was necessary to dilute the ligands to concentrations of about 2-3 µg/ml.

Reduced ligands

For each of the protein domains a mixture of 50 µl of the purified protein domain, 25 µl DTE, 500 mM, and 175 µl 10 mM Tris-HCl was prepared, and incubated at 37°C for 30 minutes. The ligand was then diluted in 10 mM acetic acid to obtain a suitable concentration for immobilization.

3.10.4 Preparation of analytes

The analytes were serially diluted in running buffer to at least five concentrations, with the dilution factor 2 between each concentration. As recommended in Biacore Advisor Kinetics [21] the concentration ranges were adjusted so that the lowest concentration showed measurable binding and at least one of the higher concentrations reached steady state during sample injection. This corresponded to concentrations ranging from about 0.01 to 1 µM.

For reliable interaction analyses with SPR it is necessary that the analyte buffer has the same composition as the running buffer [21]. In order to achieve this, the analytes with a stock concentration less than about five times higher than the highest concentration wanted was buffer exchanged into the running buffer over the columns illustra NAP™-5 and illustra NAP™-10.

3.10.5 Reference surfaces

In the first experiments the reference surfaces were immobilized with a dimer of the albumin binding domain (ABD) of streptococcal protein G. Hereby, subtraction of any interactions with the ABD of the fusion proteins was enabled. The ABD dimer was immobilized in the same way as the other ligand molecules. Since no such interaction was detected, the final experiments were performed with reference surfaces treated in the same way as the ligand surface but omitting the ligand.
3.10.6 IMMOBILIZATION APPROACHES

The ligands were immobilized on sensor chips CM5 (research grade). The EDC and NHS solutions were prepared according to the manufacturer’s instructions. To calculate what ligand level that gives a certain maximum analyte binding capacity Equation 1 can be used [21]. A general recommendation is to aim for a ligand level expected to give a maximum analyte binding capacity, R_max, of 500 RU [21]. For the interaction analyses of protein A domains (MW = 12 kDa) and IgG (MW=150 kDa), known to bind 1:1, this correspond to a ligand level of about 40 RU. For performance of kinetic analyses, a lower R_max (20-100 RU) is recommended [21].

Equation 1. The amount of ligand needed for a given binding capacity.

\[
\text{Ligand level} = R_{\text{max}} \cdot \frac{\text{ligand MW}}{\text{analyte MW}} \cdot \frac{1}{\text{binding stoichiometry}}
\]

Immobilization wizard “Aim for immobilized level”

In the first experiments, the immobilization procedure “Aim for immobilized level” was used for immobilization of the ligand molecules. In this software tool, wizard, the times for activation with EDC/NHS and deactivation with ethanolamine are by default set to 7 minutes, whereas the time for injection of ligand is adjusted by the software in order to reach the immobilization level specified by the user. The minimum immobilization level that can be chosen is 50 RU.

Immobilization wizard “Specify flow rate and injection time”

The wizard “Specify flow rate and injection time” gives the user the possibility to specify the flow rate and to set the time for injection of the ligand. Just as in the wizard “Aim for immobilized level” the activation time with EDC/NHS and the deactivation time with ethanolamine-HCl are by default set to 7 minutes. In the immobilization procedures where this wizard was used the injection time was set to 1 minute and the flow rate was set to 5 µl/min.

Thiol coupling

An activation solution of 80 mM 2-(2-pyridinylthio)ethaneamine hydrochloride (PDEA) and a deactivation solution consisting of 50 mM l-cysteine with 1 M NaCl were prepared according to the supplier’s instructions. To introduce reactive thiol groups to the ligand, reduction of the ligand with 50 mM DTE was performed prior to immobilization.

A method for immobilization with thiol coupling (see Appendix C) was created according to the description of the immobilization procedure in Biacore Advisor Kinetics [21]. Two minutes of activation with EDC/NHS was followed by an injection of PDEA for four minutes. After one minute of ligand injection, the surface was deactivated by injection of 50 mM l-cysteine with 1 M NaCl for seven minutes.
New method created for immobilization by amine coupling

A method for amine coupling was created that resembled the method in the wizards but gave the opportunity to adjust the activation time with EDC/NHS and also the times for ligand injection and deactivation. An example of a method created for immobilization of ligands in flow cell (Fc) 2 and 4, and activation/deactivation of Fc 1 and Fc 3 can be found in Appendix B. Immobilization using the created method was performed with activation times ranging from 60 to 180 seconds and with ligand injection times varying from 40 to 90 seconds. The time for deactivation was kept at 7 minutes in all immobilization procedures performed.

3.10.7 REGENERATION SCOUTING

The scouting procedure was carried out according to the manufacturer’s recommendations [21]. A method created for evaluation of five different regeneration solutions can be found in The method created for testing of a single regeneration solution for twenty cycles are presented in Appendix D. The regeneration solutions of 10 mM glycine-HCl at pH 3.0, 2.5, 2.0, and 1.5 respectively where purchased as ready-to-use solutions, whereas 10 mM glycine-HCl at pH 1.7 was prepared by mixing of 10 mM glycine-HCl pH 1.5 and 10 mM glycine-HCl pH 2.0 in appropriate volumes.

3.10.8 METHODS FOR INTERACTION ANALYSES

Analysis wizard “Kinetic analysis”

The wizard “Kinetic analysis” is a software tool for determination of kinetic parameters. Kinetic analyses of analytes in concentration series are performed, and kinetics constants based on 1:1 binding are automatically calculated and presented. The software used in the experiments allowed analyses with this wizard solely in Fc 2 with Fc 1 as reference surface.

New method created for interaction analyses

To be able to make use of all the flow cells and thereby reduce the number of sensor chips, new methods for kinetic analysis were created. In Appendix F the method used for the final interaction analyses are presented. By this method, interaction analyses are performed in all four flow cells and with four different analytes, each diluted into eight different concentrations. The concentration series of each analyte is run from the lowest concentration to the highest, and repeated twice. In the end of each analyte series an injection of buffer instead of analyte is performed. Prior to start of analysis of a new analyte, two start-up cycles with this analyte are performed. These start-up runs aim to stabilize the assay, and are not included in the data evaluation. Control of that the binding capacity of the sensor chip surface is maintained throughout the analyses is enabled by injection of a control analyte in between analyses with different analytes.
3.10.9 Interaction analyses with IgG fragments

Domain B, diluted to 2.4 µg/ml in 10 mM acetic acid, was immobilized by amine coupling to a sensor chip CM5 using the wizard “Specify flow rate and injection time” with an injection time of 1 minute and a flow rate of 5 µl/min. To the reference flow cell, ABD dimer diluted to 9.2 µg/ml in 10 mM acetic acid, was immobilized using the same procedure. The F\textsubscript{c} and F\textsubscript{ab} fragments, obtained from papain cleavage of antibody IgG\textsubscript{1a} (see section 3.7 and 3.8), were serially diluted in the running buffer HBS-EP. The wizard “Kinetic analysis” was used for interaction analysis.

3.11 Affinity determination

3.11.1 Interaction analyses with IgG subclasses

Affinity determination was performed for six different ligand molecules (protein A domains) to eight different IgG molecules. Immobilization of the ligands, reduced and diluted in 10 mM HAc, was performed using the immobilization method created for amine coupling (section 3.10.6) presented in Appendix B. The interaction analyses were performed using the method created for kinetic analyses (section 3.10.8 and Appendix F). In one run, the affinity determination of four different analytes to two ligands was performed. Thus, six runs were performed to analyze all 48 combinations.

3.11.2 Fitting of data to interaction models

The sensorgrams from the interaction analyses were evaluated according to the manufacturer’s recommendations [24]. The curves corresponding to the different concentrations in the dilution series of an analyte were overlaid, and the baselines of all the sensorgrams were adjusted to zero. One of the replicates of each run was chosen, and consequently curves containing disturbances such as air spikes were discarded. To compensate for any bulk effects, one of the curves obtained from the buffer injections was subtracted from all the selected curves. After adjustments of start and stop points, and selection of the data ranges to be used in the data fitting, the concentrations of the analytes were specified. The curves were subsequently fitted to one of the interaction models available in the software. The equilibrium constant, K\textsubscript{D}, and the maximum analyte binding capacity, R\textsubscript{max}, were hereby calculated and presented together with the Chi2-value. The Chi2-value is a measure of the average deviation of the experimental data from the fitted curve, and a low Chi2-value consequently indicates a better fit.
4 RESULTS AND DISCUSSION

4.1 PRODUCTION OF PROTEIN A DOMAINS

The expressed protein A domains were purified on IgG Sepharose. As an example, the chromatogram from purification of domain C, is presented in Figure 8. The domains were eluted by lowering the pH.

The purified protein A domains were collected in the eluate (F3).

The concentrations of the purified protein A domains were determined spectrophotometrically at a wavelength of 276 nm, and the total amount of produced product were calculated, see Table 7. Expression of domain E using a starting culture of the cultivation medium MDG (used for expression of the other domains), gave no product at all. When a starting culture of the cultivation medium 2 YT was used, product was expressed, but less than for the other domains.

Table 7. The total amount produced of each of the protein A domains fused to ABD.

<table>
<thead>
<tr>
<th>Protein A domain</th>
<th>Total amount (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain A</td>
<td>31</td>
</tr>
<tr>
<td>Domain B</td>
<td>55</td>
</tr>
<tr>
<td>Domain C</td>
<td>78</td>
</tr>
<tr>
<td>Domain D</td>
<td>40</td>
</tr>
<tr>
<td>Domain E</td>
<td>21</td>
</tr>
</tbody>
</table>
4.2 **Production of IgG Fragments**

*Analytical SEC*

IgG$_1$a was digested with papain for 90 minutes and an analytical SEC run was performed. The resulting chromatogram in Figure 9 shows that 87% of the antibody molecules were cleaved into F$_c$ and F$_{ab}$ fragments whereas 13% still remained as partly or non-digested molecules.

![Figure 9. Chromatogram from analytical SEC run of papain treated antibody IgG$_1$a.](image-url)
Purification on MabSelect SuRe

The IgG fragments prepared by papain treatment of antibody IgG₁α were purified on MabSelect SuRe, see Figure 10. The F\textsubscript{ab} fragments do not bind to the ligand and were consequently collected in the flow through fraction. The F\textsubscript{c} fragments together with undigested antibody molecules were eluted from the column by lowering the pH.

**Figure 10. Chromatogram from purification of IgG fragments on MabSelect SuRe.**

The F\textsubscript{ab} fragments were collected in the flow through fraction (F2) and the F\textsubscript{c} fragments, together with undigested antibody molecules, were collected in the eluate (F3).
Preparative SEC

The flow through fraction (containing F\(_{\text{ab}}\) fragments) and the eluate (containing F\(_{\text{c}}\) fragments together with undigested antibody molecules) from purification on MabSelect SuRe (see Figure 10) were further purified in a preparative SEC run, see Figure 11 and Figure 12 respectively. Fractions of 1 ml each were collected during the runs, and pooling was performed after evaluation of the chromatograms.

**Figure 11. Purification of F\(_{\text{ab}}\) fragments by preparative SEC.**
A sample of purified F\(_{\text{ab}}\) fragments was obtained by pooling the fractions A9-B6.

**Figure 12. Separation of F\(_{\text{c}}\) fragments and undigested antibody molecules in a preparative SEC run.**
A sample of purified F\(_{\text{c}}\) fragments was obtained by pooling the fractions B5-B12.
4.3 PROTEIN ANALYSIS

4.3.1 ANALYSIS OF PROTEIN A DOMAINS

MALDI-TOF

Characterization of the protein A domains fused to ABD and the monomer of the SuRe ligand, was performed by analyses with MALDI-TOF. As an example, the mass spectrum from analysis of domain E is shown in Figure 13. The results from the analyses showed that the molecular weights of all the protein A domains including the SuRe ligand corresponded well to the theoretical molecular weights, presented in Table 8.

![Mass spectrum from MALDI-TOF analysis of domain E fused to ABD.](image)

Table 8. Theoretical molecular weights of protein A domains.

<table>
<thead>
<tr>
<th>Protein A domain</th>
<th>Theoretical molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain A (fused to ABD)</td>
<td>12.5</td>
</tr>
<tr>
<td>Domain B (fused to ABD)</td>
<td>12.5</td>
</tr>
<tr>
<td>Domain C (fused to ABD)</td>
<td>12.5</td>
</tr>
<tr>
<td>Domain D (fused to ABD)</td>
<td>12.7</td>
</tr>
<tr>
<td>Domain E (fused to ABD)</td>
<td>12.1</td>
</tr>
<tr>
<td>Monomer of SuRe ligand</td>
<td>7.0</td>
</tr>
</tbody>
</table>
Microchip gel electrophoresis

To estimate the molecular weights and purity of the produced protein A domains, analysis using Agilent 2100 Bioanalyzer with Agilent Protein 80 Kit was performed. This staining technique does not enable labeling of protein A, but of the ABD domain fused to the produced domains. Consequently, the domain of the SuRe ligand, that do not contain ABD, could not be stained by this technique and is therefore not included in the analysis. The results, shown in Figure 14, show a high purity of the different domains as well as correct molecular weights of the products.

Figure 14. Results from analysis of produced protein A domains with Agilent 2100 Bioanalyzer presented in a gel-like image.
4.3.2 Analysis of IgG subclasses

The quality of the antibodies was controlled by analyses using Agilent 2100 Bioanalyzer. The results are presented in a gel-like image in Figure 15. The analyses show, as expected, that the antibodies of the IgG\textsubscript{1} and IgG\textsubscript{4} subclasses have molecular weights of about 150 kDa, and that the antibodies of subclass IgG\textsubscript{3} have a somewhat higher molecular weight, about 180 kDa. IgG\textsubscript{2}a shows a molecular weight higher than the expected for antibodies of the IgG\textsubscript{2} subclass, and it also generates a broad band on the gel. The fusion protein Etanercept, with a theoretical molecular weight of 150 kDa, contains a compound with an apparent molecular weight of about 200 kDa.

![Figure 15. Results from analysis of antibodies with Agilent 2100 Bioanalyzer presented in a gel-like image.](image-url)
IgG fragments

To obtain Fc and Fab fragments respectively the antibody IgG1a was digested with papain. The purified fragments, together with samples of the papain treated antibody and the undigested antibody were analyzed by SDS-PAGE. The results, presented in Figure 16, show that most of the antibody molecules are digested by the papain treatment. The obtained Fc and Fab fragments have, as expected, a molecular weight of about 50 kDa. Some of the Fab fragments are reduced into H chains and L chains.

**Figure 16.** SDS-PAGE of Cy5 labeled antibody IgG1a and its fragments.
1) Fab fragments purified on MabSelect SuRe and by SEC.
2) Remaining non-digested antibody from preparation of IgG fragments, purified on MabSelect SuRe and by SEC.
3) Fc fragments purified on MabSelect SuRe and by SEC.
4) Papain treated antibody IgG1a.
5) Non-digested antibody IgG1a.
6) Reduced polyclonal IgG.
4.4 OPTIMIZATION OF BIACORE EXPERIMENTS

4.4.1 FITTING OF DATA TO INTERACTION MODELS

The data obtained from the primary interaction analyses showed a poor fit to the 1:1 interaction model, whereas the fitting to the model Bivalent analyte as well as the model Heterogeneous ligand was significantly better (see Figure 17).

The ligand used is domain B of protein A, the analyte is antibody IgG\textsubscript{1a}.

Fitting of the same data to the interaction model Bivalent analyte resulted in a Chi\textsuperscript{2} of 6.96, and the corresponding Chi\textsuperscript{2} value for the model Heterogeneous ligand was 10.4.

In order to evaluate whether F\textsubscript{ab} interactions could be a contributive reason to the poor fit to the 1:1 interaction model, interaction analyses were performed with F\textsubscript{ab} fragments and F\textsubscript{c} fragments respectively as the analyte molecules. The evaluated sensorgrams from these analyses can be seen in Figure 18. The F\textsubscript{c} fragments bind to the ligand molecules of domain B of protein A, whereas the F\textsubscript{ab} fragments do not. Thus, F\textsubscript{ab} interactions do not contribute to the affinity between domain B of protein A and the antibody IgG\textsubscript{1a} and is consequently not the reason for the poor fitting to the 1:1 interaction model.
In an attempt to control the orientation of the ligands and thereby obtain a better fit to the 1:1 interaction model, thiol coupling was used for immobilization. By coupling via the C-terminal cysteine of the ligand, the molecules are hereby immobilized in a directed manner. A comparison of results from kinetic analyses performed on sensor chips immobilized with thiol coupling and amine coupling respectively can be seen in Figure 19. The fit to the 1:1 interaction model were not significantly better when thiol coupling had been used for immobilization. Since thiol coupling, in addition, is a more time consuming coupling method than amine coupling, no further experiments were performed using this immobilization technique.

The \( R_{\text{max}} \)-values calculated from the primary experiments were typically in the range of 100-150 RU. To be able to achieve even lower ligand levels, and thereby lower the \( R_{\text{max}} \)-values, it was necessary to use a shorter time of activation with EDC/NHS than possible in the immobilization wizards. A new method (shown in Appendix B) for immobilization, that enabled this, was therefore created.

As shown in Figure 20, a low ligand level resulted in a significantly better fit (lower Chi2-value) to the 1:1 interaction model than a high ligand level did. Consequently, the following analyses were performed on sensor chips immobilized to a ligand level low enough to give \( R_{\text{max}} \)-values of 20-100 RU (see 3.10.6), preferably in the lower area of this interval.
- Results and Discussion -

Figure 20. Comparison of kinetic analyses performed on sensor chips immobilized to different levels.

a) Ligand: low level of domain B of protein A  
Analyte: antibody IgG  
$R_{\text{max}}=21.7$  
Chi2=1.52

b) Ligand: high level of domain B of protein A  
Analyte: antibody IgG  
$R_{\text{max}}=118$  
Chi2=67.4

4.4.2 EVALUATION OF BASELINE STABILITY

Decreasing baseline levels were observed throughout the interaction analyses. A possible explanation was that the ligand molecules to a great extent were dimerized and that monomer units leached during the analyses. All the ligands have free thiol groups, and can consequently form dimers via the thiols. During injection of analyte there was a possibility of thiol reduction and thereby loss of mass from the sensor chip surface. In order to ensure that the ligand molecules were immobilized as monomers, a reduction of the protein domains were performed just before immobilization.

After these experiments had been performed, information from Biacore AB [23] (now a part of GE Healthcare) stated that a descending baseline throughout interaction analysis is often observed. The explanation to this phenomenon is that small modifications of the dextran matrix on the sensor chip surface occur during the analyses. This theory was supported by the fact that descending baseline levels were observed also in the reference surfaces where no ligand had been immobilized.

4.4.3 REGENERATION SCOUTING

As a sensor chip surface is used for series of measurements it is necessary to regenerate the surface after each analysis cycle. Under optimal regeneration conditions, any analyte still bound to the ligand after an analysis cycle is removed whereas the ligand is left unaffected. To establish the optimal regeneration procedure for an assay it is important to perform a regeneration scouting procedure where the effects of different regeneration conditions are tested. The regeneration scouting started with testing of four different regeneration solutions (10 mM glycine-HCl at pH of 3.0, 2.5, 2.0 and 1.7) for five cycles each. Each cycle consisted of an analyte injection followed by a pulse of regeneration solution. The solutions were injected in order from the one that gives the gentlest conditions (pH 3.0) and proceed successively to harsher conditions (pH 1.7). The result can be found in Figure 21.
Figure 21. Evaluation of regeneration scouting with four different regeneration solutions. Four different regeneration solutions, 10 mM glycine-HCl at different pH values, were tested for five cycles each. The most stable levels of both baseline (a) and analyte binding (b) were obtained with 10 mM glycine-HCl, pH 1.7. The most stable levels for baseline as well as for analyte binding were obtained with the regeneration solution 10 mM glycine-HCl, pH 1.7. Both analyte response and baseline are also close to the starting values after each cycle. Another twenty regeneration cycles with this regeneration solution (Figure 22) made it clear that this solution could be used in the interaction analyses.

Figure 22. Evaluation of regeneration scouting with one regeneration solution used in twenty cycles. The baseline level (a) as well as the level of binding analyte (b) is stable when regeneration is performed with 10 mM glycine-HCl, pH 1.7.
4.5 DETERMINATION OF $K_D$-VALUES

Analyses of the interactions between the six protein A domains and the eight antibodies/Fc-fusion protein were performed, and the resulting sensorgrams were evaluated. In Table 9 the $K_D$-value, expressed in nM, for each of the 48 combinations is presented together with the $R_{\text{max}}$-value of the analysis.

Table 9. $K_D$-values, expressed in nM, obtained from interaction analyses of protein A domains to IgG.
The values in parenthesis are considered to not be reliable due to uncertainty of the quality IgG$_2$a and a different behaviour of domain E compared to the other domains.

<table>
<thead>
<tr>
<th></th>
<th>Domain A</th>
<th>Domain B</th>
<th>Domain C</th>
<th>Domain D</th>
<th>Domain E</th>
<th>Monomer of SuRe ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG$_1$b</td>
<td>15</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>(50)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>$R_{\text{max}}=30$</td>
<td>$R_{\text{max}}=43$</td>
<td>$R_{\text{max}}=40$</td>
<td>$R_{\text{max}}=27$</td>
<td>$R_{\text{max}}=7$</td>
<td>$R_{\text{max}}=50$</td>
</tr>
<tr>
<td>IgG$_1$c</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td>(17)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>$R_{\text{max}}=75$</td>
<td>$R_{\text{max}}=68$</td>
<td>$R_{\text{max}}=65$</td>
<td>$R_{\text{max}}=45$</td>
<td>$R_{\text{max}}=21$</td>
<td>$R_{\text{max}}=50$</td>
</tr>
<tr>
<td>IgG$_2$a</td>
<td>(200)</td>
<td>(100)</td>
<td>(80)</td>
<td>(200)</td>
<td>(200)</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>$R_{\text{max}}=9$</td>
<td>$R_{\text{max}}=16$</td>
<td>$R_{\text{max}}=20$</td>
<td>$R_{\text{max}}=9$</td>
<td>$R_{\text{max}}=1$</td>
<td>$R_{\text{max}}=18$</td>
</tr>
<tr>
<td>IgG$_3$a</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>IgG$_3$b</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>IgG$_4$a</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>(15)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$R_{\text{max}}=33$</td>
<td>$R_{\text{max}}=44$</td>
<td>$R_{\text{max}}=40$</td>
<td>$R_{\text{max}}=26$</td>
<td>$R_{\text{max}}=4$</td>
<td>$R_{\text{max}}=45$</td>
</tr>
<tr>
<td>IgG$_4$b</td>
<td>40</td>
<td>20</td>
<td>15</td>
<td>15</td>
<td>(80)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>$R_{\text{max}}=24$</td>
<td>$R_{\text{max}}=35$</td>
<td>$R_{\text{max}}=32$</td>
<td>$R_{\text{max}}=22$</td>
<td>$R_{\text{max}}=4$</td>
<td>$R_{\text{max}}=35$</td>
</tr>
<tr>
<td>Etanercept</td>
<td>50</td>
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<td>20</td>
<td>35</td>
<td>(400)</td>
<td>15</td>
</tr>
<tr>
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<td>$R_{\text{max}}=18$</td>
<td>$R_{\text{max}}=32$</td>
<td>$R_{\text{max}}=23$</td>
<td>$R_{\text{max}}=18$</td>
<td>$R_{\text{max}}=7$</td>
<td>$R_{\text{max}}=35$</td>
</tr>
</tbody>
</table>

N.D. = not detectable

None of the two antibodies of the IgG$_3$ subclass shows detectable interaction with any of the protein A domains. This result is in accordance with previous reports [11].

IgG$_1$a showed a significantly lower affinity to protein A domains than the other antibodies. Analysis with microchip gel electrophoresis of this antibody indicated bad quality of the product. This is probably a contributive reason to the low affinity to the protein A domains.
Domain E showed low binding to all IgG subclasses. In addition, this domain was not expressed when the cultivation medium MDG, used for expression of the other domains, were used in the starting culture. When the cultivation medium 2 YT was used product was expressed, but the resulting expression level was significantly lower than for the other domains. Also at immobilization onto the sensor chip this domain distinguished from the other domains. To reach a certain immobilization level, longer activation times and higher ligand concentrations had to be used. However, analyses by MALDI-TOF and by microchip gel electrophoresis of this domain did not indicate any quality issues.

The reliability of the equilibrium constants obtained is affected by the purity of the analytes, as well as by the accuracy of the analyte concentrations. Since the extinction coefficients were not known for all the antibodies used in the interaction analyses, the exact concentrations of the analytes were difficult to determine. This gives a certain degree of uncertainty to the obtained $K_D$-values, calculated by the software based on the analyte concentrations specified, and can contribute to the differences in equilibrium constants for antibodies of the same subclass.
5 CONCLUSIONS

The aim of this project was to determine the affinity of individual protein A domains to different IgG subclasses using surface plasmon resonance. The IgG-binding protein A domains E, D, A, B, and C were expressed as fusion proteins to ABD in *E. coli*. In addition to these five domains, a monomer of the engineered ligand used in protein A medium MabSelect Sure was included in the study. The domains were immobilized onto sensor chips CM5 using amine coupling, and seven different antibodies as well as one Fc-fusion protein were used as analytes.

- To obtain a good fit (low chi2-value) of the data from kinetic analyses to the 1:1 interaction models, it is of great importance to use a low ligand level. \( R_{\text{max}} \)-values of about 20, or somewhat higher, results in the best fits.

- In analyses of the interactions between protein A domains and IgG subclasses, appropriate regeneration is achieved by use of 10 mM glycine-HCl, pH 1.7. By use of this regeneration solution, the baseline as well as the analyte binding capacity was kept at a stable level throughout the analyses.

- Early data from kinetic analyses showed poor fitting (high chi2-value) to 1:1 interaction models but significantly better fitting to a bivalent interaction model. This was shown not to be due to F\(_{ab}\) interactions. No significant difference in fitting was noted when immobilization was performed by thiol coupling instead of amine coupling.

- All human IgG subclasses except IgG\(_3\) bind to all protein A domains including the monomer of the SuRe ligand.

- For IgG\(_1\) and IgG\(_4\), there are no significant differences in the affinity to any of the protein A domains except for domain E. For unknown reason this domain behave differently than the other domains when it comes to immobilization onto sensor chip surfaces and binding to IgG. The equilibrium constants, \( K_D \)-values, obtained for IgG\(_1\) and IgG\(_4\) were in the low nanomolar range.

- The \( K_D \)-values of IgG\(_2\) could not be determined with any reliability due to quality issues of the commercial antibody used. However, protein A chromatography is used for large scale purification of monoclonal antibodies of human IgG\(_2\) and there is no reason to believe that IgG\(_2\) has lower affinity to protein A than IgG\(_1\) and IgG\(_4\).
FUTURE STUDIES

- In order to obtain more reliable $K_D$ values for the interactions between protein A domains and IgG subclasses it would be necessary to repeat the performed Biacore experiments. The antibody of the IgG$_2$ subclass as well as the protein A domain E should be replaced with products of higher purity or better quality.

- To obtain an even wider knowledge of the interactions between protein A domains and IgG subclasses, the study could be complemented with analyses of IgG subclasses from species other than human.

- To investigate the reason for the different behaviour of domain E, compared to the other domains, concerning immobilization onto the sensor chip and the binding to IgG subclasses further studies would be necessary
ACKNOWLEDGEMENTS

I would like to thank…

- Tomas Björkman and Gustav Rodrigo, my supervisors, for giving me the opportunity to perform my master thesis project at GE Healthcare, and for all the support and assistance throughout the project.

- Karin Nord at Affibody AB for kindly donating the cell-lines for expression of the protein A domains.

- Jozsef Vasi and Frida Lindström for all the help and assistance in the fermentation lab.

- Susanne Nyholm-Westin for introducing me to the Biacore instrument and for helping me getting started with the Biacore experiments.

- Anita Larsson for valuable advice concerning the Biacore experiments, and for fast and informative answers to my questions.

- Anna Grönberg for kindly sharing pictorial material concerning the SPR technology and the Biacore instrumentation.

- Helen Hillmering, my opponent, for carefully reading through this report and giving me valuable feedback.

- Bo Liedberg for comments and feedback on my work and this report.

- Andreas Muranyi for introducing me to the department and the company, and for excellent administrative support during the project.

- Everyone at Antibody Processing for help and company along the way.
REFERENCES

APPENDIX A:
Medium composition

Auto inducer medium for clones with T7 promoter (host: BL21 DE(3) or similar)
ZYP-5052:

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$ × 2 H$_2$O</td>
<td>8.9</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>6.8</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>3.3</td>
</tr>
<tr>
<td>Glycerol (of 87% w/v stock)</td>
<td>5.75 ml</td>
</tr>
</tbody>
</table>

Components are dissolved in Milli-Q water and the solution is sterilized in an autoclave at 121°C for 20 minutes.

After sterilization add the following sterile compounds:

- MgSO$_4$×7H$_2$O (of 1.2 M sterile stock) 1.7 ml
- Trace metal solution* 0.61 ml
- CaCl$_2$× 2 H$_2$O (0.14 M sterile stock in 1.2 M HCl) 0.61 ml
- Glucose (of 60% w/v sterile stock) 0.84 ml
- α-D-Lactose monohydrate (of 20 % w/v sterile stock) 10 ml
- Vitamin solution** 0.6 ml

Non-inducing medium for stock/seed production of the above clones
MDG:

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$ × H$_2$O</td>
<td>8.9</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>6.8</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>2.67</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Components are dissolved in Milli-Q water and the solution is sterilized in an autoclave at 121°C for 20 minutes.

After sterilization add the following sterile compounds:

- MgSO$_4$ × 7H$_2$O (of 1.2 M sterile stock) 1.7 ml
- Trace metal solution* 0.61 ml
- CaCl$_2$ × 2 H$_2$O (0.14 M sterile stock in 1.2 M HCl) 0.61 ml
- Glucose (of 60% sterile stock) 8.4 ml
- Aspartic acid Na salt (10 % w/v) 25 ml
*Trace metal solution:*

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron chloride, FeCl$_3$ $\times$ 6 H$_2$O</td>
<td>53</td>
</tr>
<tr>
<td>Zinc sulphate, ZnSO$_4$ $\times$ 7 H$_2$O</td>
<td>16</td>
</tr>
<tr>
<td>Cobalt chloride, CoCl$_2$ $\times$ 6 H$_2$O</td>
<td>4</td>
</tr>
<tr>
<td>Copper sulphate, CuSO$_4$ $\times$ 5 H$_2$O</td>
<td>4</td>
</tr>
<tr>
<td>Boric acid, H$_3$BO$_3$</td>
<td>4</td>
</tr>
<tr>
<td>Manganese sulphate, MnSO$_4$ $\times$ 1 H$_2$O</td>
<td>20</td>
</tr>
</tbody>
</table>

Components are dissolved in 1.2 M HCl and the solution is sterilized in an autoclave at 121°C for 20 minutes.

**Vitamin solution:**

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-pantothenic acid, calcium salt, C$<em>{18}$H$</em>{32}$O$_{10}$N$_2$Ca</td>
<td>0.5</td>
</tr>
<tr>
<td>Choline chloride, C$<em>{5}$H$</em>{14}$C$_{2}$NO</td>
<td>0.5</td>
</tr>
<tr>
<td>Folic acid, C$<em>{10}$H$</em>{10}$N$_7$O$_6$</td>
<td>0.5</td>
</tr>
<tr>
<td>Myo-Inositol, C$<em>{6}$H$</em>{12}$O$_6$</td>
<td>1.0</td>
</tr>
<tr>
<td>Niacinamide, C$<em>{6}$H$</em>{9}$N$_2$O</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxal hydrochloride, C$<em>{5}$H$</em>{9}$NO$_3$ $\times$ HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Riboflavin, C$<em>{17}$H$</em>{20}$N$_4$O$_6$</td>
<td>0.05</td>
</tr>
<tr>
<td>Tiamin hydrochloride, C$<em>{12}$H$</em>{18}$C$_{12}$N$_4$OS $\times$ X H$_2$O</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Components are dissolved in Milli-Q water and filter sterilized (0.2 μm).

**Cultivation medium 2YT:**

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone S</td>
<td>16</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
</tbody>
</table>

Components are dissolved in Milli-Q water and the solution is sterilized in an autoclave at 121°C for 20 minutes.
APPENDIX B:
Biacore method for amine coupling

Immobilization by amine coupling is performed in Fc 2 and Fc 4. The reference flow cells, Fc 1 and Fc 3, are activated and deactivated but ligand is omitted.

```
main
prime
normalize r2f2

detection 1
aprog immobilization_Fc1
detection 2
aprog immobilization_Fc2
detection 3
aprog immobilization_Fc3
detection 4
aprog immobilization_Fc4

append standby
end

!Fc1
define aprog immobilization_Fc1
flow 10
flowpath 1
transfer r2a1 r2a3 50
transfer r2a2 r2a3 50
mix r2a3 80
* inject r2a3 15 !90 s activation
-15 rpoint baseline -b
100 rpoint EDC/NHS
flow 5
inject r2a5 35 !7 min deactivation
590 rpoint ethanolamine
end

!Fc2
define aprog immobilization_Fc2
flow 10
flowpath 2
transfer r2b1 r2b3 50
transfer r2b2 r2b3 50
mix r2b3 80
* inject r2b3 15 !90 s activation
-15 rpoint baseline -b
* inject r2b4 10 !60 s ligand injection

!Fc3
define aprog immobilization_Fc3
flow 10
flowpath 3
transfer r2c1 r2c3 50
transfer r2c2 r2c3 50
mix r2c3 80
* inject r2c3 15 !90 s activation
-15 rpoint baseline -b
100 rpoint EDC/NHS
flow 5
inject r2c5 35 !7 min deactivation
590 rpoint ethanolamine
end

!Fc4
define aprog immobilization_Fc4
flow 10
flowpath 4
transfer r2d1 r2d3 50
transfer r2d2 r2d3 50
mix r2d3 80
* inject r2d3 15 !90 s activation
-15 rpoint baseline -b
* inject r2d4 10 !60 s ligand injection
-15 rpoint EDC/NHS -b
110 rpoint ligand
250 rpoint ligand_2
flow 5
inject r2d5 35 !7 min deactivation
690 rpoint ethanolamine
end
```
APPENDIX C:
Biacore method for thiol coupling

Immobilization by thiol coupling is performed in Fc 4.

main
detection 4
aprog thiolcoupling_Fc4
append standby
end

define aprog thiolcoupling_Fc4
flow 5
flowpath 4
transfer r2d1 r2d3 40
transfer r2d2 r2d3 40
mix r2d3 60
* inject r2d3 10 !2 min EDC/NHS
  -15 rpoint baseline -b
* inject r2d4 20 !4 min PDEA
  -15 rpoint EDC/NHS -b
* inject r2d5 5 !1 min ligand injection
  -15 rpoint PDEA -b
  80 rpoint ligand
inject r2d6 35 !7 min deactivation
450 rpoint cysteine/NaCl
end
APPENDIX D:
Biacore method for 5*5 cycles of regeneration scouting

Regeneration scouting is performed in flow cell 1 and 2 with five different regeneration solutions run for five cycles each. Before every injection of regeneration solution an analyte injection is performed.

main
detection 1,2
loop regscout order
aprog regscout_IgG %caption %analytpos %regpos
endloop
append standby
end

!---------------------------------------
define aprog regscout_IgG
param %caption %analytpos %regpos
caption %caption
flow 10
flowpath 1,2

!analyt inj
* inject %analytpos 30 !3 min injection
-15 rpoint baseline -b
200 rpoint analyt

!reg inj
flow 40
* inject %regpos 20 !0.5 min injection
45 rpoint regenerering

end

!---------------------------------------
define loop regscout
lparam %caption %analytpos %regpos
times 5
glycine_pH3 r2a1 r2b1
glycine_pH2.5 r2a1 r2b2
glycine_pH2 r2a2 r2b3
glycine_pH1.7 r2a2 r2b4
glycine_pH1.5 r2a3 r2b5
end
APPENDIX E:
Biacore method for 20 cycles of regeneration scouting

Regeneration scouting is performed in Fc 3 with one regeneration solution for twenty cycles. Before every injection of regeneration solution an analyte injection is performed.

main
detection 3
loop regscout order
aprog regscout_IgG_20 %caption %analytpos %regpos
endloop
append standby
end

!---------------------------------------
define aprog regscout_IgG_20
param %caption %analytpos %regpos
caption %caption
flow 10
flowpath 3

!analyt inj
* inject %analytpos 30 !3 min injektion
-15 rpoint baseline -b
200 rpoint analyt

!reg inj
flow 40
* inject %regpos 20 !0.5 min injektion
45 rpoint regenerering
end

!---------------------------------------
define loop regscout
lparam %caption %analytpos %regpos
times 5

glycine_pH1.7 r2a1 r2b1
glycine_pH1.7 r2a2 r2b2
glycine_pH1.7 r2a3 r2b3
glycine_pH1.7 r2a4 r2b4
end
APPENDIX F:
Biacore method for kinetic analysis

Interaction analyses are performed in flow cell 2 and 4, with flow cell 1 and 3 used as reference surfaces. Four different analytes, diluted into eight concentrations each, are used. The analytes are injected in series, from the lowest concentration to the highest, repeated twice. A buffer injection is performed in the end of each analyte series. Prior to injection of a new analyte two start-up cycles are carried out. Injection of a control analyte between every analyte series enables control of the binding capacity of the sensor chip surface throughout the analyses.

```plaintext
main
prime
normalize r2f2
detection 2-1,4-3

aprog control %analyt %analytpos %regpos

! ANALYTE1
loop start_up1 order
  aprog kinetic %analyt %analytpos %regpos endloop
loop analyte1 step
  aprog kinetic %analyt %analytpos %regpos endloop

aprog control %analyt %analytpos %regpos

! ANALYTE2
loop start_up2 order
  aprog kinetic %analyt %analytpos %regpos endloop
loop analyte2 step
  aprog kinetic %analyt %analytpos %regpos endloop

aprog control %analyt %analytpos %regpos

! ANALYTE3
loop start_up3 order
  aprog kinetic %analyt %analytpos %regpos endloop
loop analyte3 step
  aprog kinetic %analyt %analytpos %regpos endloop

aprog control %analyt %analytpos %regpos

! ANALYTE4
loop start_up4 order
  aprog kinetic %analyt %analytpos %regpos endloop
```

```plaintext
loop analyte4 step
  aprog kinetic %analyt %analytpos %regpos endloop

aprog control %analyt %analytpos %regpos

append standby
end

!-------------------------------------
define aprog kinetic
param %analyt %analytpos %regpos
caption %analyt
flow 30
flowpath 1,2,3,4

!analyt inj
* kinject %analytpos 90 900 !3 min injection,
  15 min dissociation
-15 rpoint baseline -b
170 rpoint binding1
1070 rpoint binding2

!reg inj
* inject %regpos 15 !0.5 min injection
70 rpoint regenerering

end
!-------------------------------------
```
define aprog control
param %analyt %analytpos %regpos
caption %analyt
flow 30
flowpath 1,2,3,4

!analyt inj
* kinject r2f1 90 900 !3 min injection, 15 min
dissociation
-15 rpoint baseline -b
170 rpoint binding1
1070 rpoint binding2

!reg inj
* inject r2e10 15 !0.5 min injection
70 rpoint regenerering
end

!-------------------------------------

!START-UP1
define loop start_up1
lparam %analyt %analytpos %regpos
times 2
start-up1 r2a10 r2e9
end

!START-UP2
define loop start_up2
lparam %analyt %analytpos %regpos
times 2
start-up2 r2b10 r2e9
end

!START-UP3
define loop start_up3
lparam %analyt %analytpos %regpos
times 2
start-up3 r2c10 r2e9
end

!START-UP4
define loop start_up4
lparam %analyt %analytpos %regpos
times 2
start-up4 r2d10 r2e9
end

!-------------------------------------

!ANALYTE1
define loop analyte1
lparam %analyt %analytpos %regpos
times 2
1Konc8 r2a8 r2e1
1Konc7 r2a7 r2e1
1Konc6 r2a6 r2e1
1Konc5 r2a5 r2e1
1Konc4 r2a4 r2e2
1Konc3 r2a3 r2e2
1Konc2 r2a2 r2e2
1Konc1 r2a1 r2e2
1buffert r2a9 r2e2
end

!ANALYTE2
define loop analyte2
lparam %analyt %analytpos %regpos
times 2
2Konc8 r2b8 r2e3
2Konc7 r2b7 r2e3
2Konc6 r2b6 r2e3
2Konc5 r2b5 r2e3
2Konc4 r2b4 r2e4
2Konc3 r2b3 r2e4
2Konc2 r2b2 r2e4
2Konc1 r2b1 r2e4
2buffert r2b9 r2e4
end

!ANALYTE3
define loop analyte3
lparam %analyt %analytpos %regpos
times 2
3Konc8 r2c8 r2e5
3Konc7 r2c7 r2e5
3Konc6 r2c6 r2e5
3Konc5 r2c5 r2e5
3Konc4 r2c4 r2e6
3Konc3 r2c3 r2e6
3Konc2 r2c2 r2e6
3Konc1 r2c1 r2e6
3buffert r2c9 r2e6
end

!-------------------------------------
!ANALYTE4
define loop analyte4
lparam %analyt %analytpos %regpos
times 2
4Konc8 r2d8 r2e7
4Konc7 r2d7 r2e7
4Konc6 r2d6 r2e7
4Konc5 r2d5 r2e7
4Konc4 r2d4 r2e8
4Konc3 r2d3 r2e8
4Konc2 r2d2 r2e8
4Konc1 r2d1 r2e8
4buffert r2d9 r2e8
end
!-----------------------------