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Conformational changes of antibodies upon adsorption onto hydrophobic interaction chromatography surfaces

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ABSTRACT

Differential scanning calorimetry was established for in-situ measurement of the transition temperatures of antibodies when adsorbed on hydrophobic interaction chromatography media. This method is also suitable for non-transparent media, which is an advantage over spectroscopic methods that cannot be used in many cases due to large background signals. The three transition temperatures of an antibody were lowered when the molecule was adsorbed onto Phenyl and Butyl functionalized Toyopearl particles as well as on Phenyl Sepharose 6 Fast Flow when bound at moderate to high salt concentration compared to the values in free solution. The first two melting points, representing the CH2 domain and the Fab fragment, are more affected than the highest melting point, which corresponds to the CH3 domain. It is obvious that domains which are less stable are more likely to undergo conformational change upon adsorption. It could be shown that the conformational changes occurring in antibodies upon adsorption to HIC media are directly proportional to the hydrophobicity of the stationary phase and that they are reversible. Upon elution, the protein returns to its original conformation. For all four tested resins, a negative value for both ΔH as well as ΔS was calculated, leading to opposing contributions to ΔG.

1. Introduction

Hydrophobic interaction chromatography (HIC) is frequently used because it is an orthogonal method to ion-exchange and affinity chromatography with a completely different selectively. One of the most common arguments for using HIC is that this method is widely regarded as a "non-destructive technique" due to the absence of organic modifiers and harsh elution conditions [1]. It is commonly believed that proteins can be purified and analyzed in their native condition by hydrophobic interaction chromatography. Still, this claim has to be taken with a grain of salt, since over the years many studies have at first suspected and later outright proven, that conformational changes can occur during the adsorption of proteins onto hydrophobic surfaces [2–6]. Our group proposed a model, according to which a certain fraction of the injected protein unfolds upon adsorption onto the HIC stationary phases resulting in stronger binding and delayed elution from the column [7,8], which would explain the loss of protein often observed when HIC is used in a purification step [9]. The partial unfolding has been measured in-situ by Attenuated Total Reflectance Fourier Transformed Infrared (ATR FT-IR) spectroscopy. Recently, Antos and coworkers [10] suggested that assuming a reversible unfolding mechanism might be more accurate for describing the behavior of certain proteins during adsorption in HIC and supported this claim by Nano Differential Scanning Fluorimetry measurements.

While these conformational changes upon adsorption have readily been observed for certain proteins, others do not seem to be affected. It has been hypothesized that this tendency towards unfolding upon adsorption is strongly dependent on the type of the stationary phase used on the one hand and on the structure and the physical properties of the protein in question on the other hand. Especially the adiabatic compressibility of the protein has been suspected to be of decisive influence [8], since it has been observed that "softer" proteins, which have higher adiabatic compressibility, are more prone towards unfolding and show stronger retention on hydrophobic surfaces [11].

Most of the available data on this topic has been obtained using classical model proteins including lysozyme, bovine serum albumin (BSA), β-lactoglobulin, Ca^{++} depleted lactalbumin or ovalbumin. This raises the question, if and how molecules that are of higher interest for the biopharmaceutical sector, such as antibodies, are affected by the interaction with hydrophobic surfaces that occurs in HIC. In order to tackle this, we chose a GMP manufactured IgG1 therapeutic antibody as the model protein for this study, which is a good representative of the majority of molecules in this class of bio-

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therapeutics and compared the adsorption behavior of this protein towards various commercially available HIC media.

In the past, ATR FT-IR has been used for studying conformational changes during the adsorption of proteins onto chromatographic surfaces. This method, however, is only partially suitable for the purpose, since the measurement is heavily dependent on the optical characteristics of the stationary phase particles. While good results could be achieved for Sepharose-based materials, polymetacrylate particles could not be analyzed at all due to a lack of translucency and high background signals [8]. Based on the nature of calorimetric measurements, it can be expected that DSC measurements are less sensitive to these factors.

As analysis methods for studying the adsorption behavior of our model antibodies, Isothermal Titration Calorimetry (ITC) and Differential Scanning Calorimetry (DSC) were chosen. ITC is a well-established method for quantifying the thermodynamic parameters associated with the interaction of proteins with all sorts of ligands and chromatographic stationary phase particles [12–14]. The original method for this was developed by Chen et al. [15–18]. In such an ITC experiment, the heat flow resulting from repeated injections of protein solution into the measurements cell containing stationary phase slurry is monitored. Based on this heat flow and on the protein concentration adsorbed per unit stationary phase, which can be calculated from the adsorption isotherm, it is then possible to calculate the adsorption enthalpy ΔH_{ads}. As such ITC is the only method that allows direct measurement of the enthalpy of adsorption of proteins binding onto chromatographic stationary phases. As structural changes of a protein generally require energy in order to occur, a larger extent of conformational changes under certain conditions should also manifest itself in a change in ΔH_{ads}, since the unfolding process requires energy. Especially when conformational changes are involved, enthalpy measurements by ITC have been shown to yield more consistent results than van't Hoff analysis [19].

Differential Scanning Calorimetry on the other hand, allows a more direct detection of conformational changes. The thermal unfolding process of proteins occurs over a narrow temperature window. Thus, it is possible to reliably determine the transition midpoint between folded and unfolded state, also referred to as the melting temperature, by measuring the heat absorbed or released by a sample upon controlled heating in a DSC instrument. This temperature value is commonly used as a reference number for protein stability and any differences between the protein in free solution and the protein in the adsorbed state would strongly indicate conformational changes. For antibodies, usually 2–3 different transition peaks are observed in DSC experiments, representing the unfolding events of the different domains of the molecule. The lowest transition temperature can be attributed to the unfolding of the CH_2 domain, the second one corresponds to the antigen-binding fragment (Fab) and the third one indicates the unfolding of the CH_3 domain. For different antibodies, these peaks may however overlap to varying degrees [20–22].

For this study, the thermal stability of the model antibody was analyzed in free solution and in the adsorbed state as well as after adsorption and subsequent elution from a stationary phase by DSC. Additionally, the thermodynamic quantities ΔG, ΔH and ΔS associated with the binding of the antibody onto the stationary phases were measured by ITC.

2. Materials and methods

2.1. Materials and chemicals

All chemicals were of analytical grade, unless stated otherwise. Disodium hydrogen phosphate (1.06580.500) and ammonium sulfate for buffer preparation (1.0217.500) were purchased from Merck.

The recombinant monoclonal antibody CH14.18 was kindly provided by APEIRON biologics. It is a mouse-human chimeric IgG1, produced in Chinese Hamster Ovary (CHO) cells [23].

TOYOPEARL Phenyl–650 M (0019818) and TOYOPEARL Butyl–650 M (0019802) were purchased from Tosoh Bioscience and are both based on hydroxylated polymethacrylic polymer beads, Butyl-S Sepharose 6 Fast Flow (17-0978-10), Phenyl Sepharose 6 Fast Flow (High Sub) (17-0973-10) and Phenyl Sepharose 6 Fast Flow (Low Sub) (17-0965-10) were obtained from GE Healthcare Life Sciences.

Decon Labs Decon 90 was purchased from Fisher Scientific (11761168).

2.2. Differential Scanning Calorimetry (DSC)

For all protein samples, a buffer exchange via ultra-diafiltration was performed using the corresponding experiment buffer (20 mM phosphate containing either 0 mM, 400 mM or 800 mM ammonium sulfate at pH 7.3) as ultra-diafiltration buffer, Amicon Ultra-15 Centrifugal Filter Units with a cut-off of 50 kDa (Merck Millipore UFC905096) and a Heraeus Multifuge X3 FR centrifuge (Thermo Scientific).

For the antibody in free solution measurements, the protein was then diluted to a concentration of 0.25 mg/mL. 650 μL of this solution were loaded into the sample cell of a TA- Instruments Nano DSC instrument (model: 602000), the reference cell was filled with buffer and a thermoscan from 25 °C to 100 °C with a scan rate of 1 °C/min was performed. The obtained thermogram data was then analyzed using the TA Instruments NanoAnalyse software and a two-state scaled fitting model with 3 peaks.

For the measurements of antibody adsorbed onto chromatographic stationary phase particles, a sample volume corresponding to 180 μg of the model antibody in the chosen experiment buffer was added to 350 μL of a 50% slurry of the stationary phase in the corresponding experiment buffer and further diluted to a total volume of 700 μL. This solution was then incubated under end-over-end shaking for 4 h. To remove any non-adsorbed antibody, the sample was then spinned down in a Fisherbrand HS10022 mini-centrifuge, the excess liquid was removed and the particles were washed 3 times with an equal amount of the corresponding experiment buffer. The suspension of stationary phase particles with adsorbed protein in experiment buffer was then loaded into the sample cell of the Nano DSC and measured using the same conditions as for the antibody in free solution.

For the blank measurements with stationary phase particles, but without protein, a 0.25% slurry of the stationary phase in the corresponding experiment buffer was prepared and loaded into the Nano-DSC.

In between sample runs, the instrument was cleaned by flushing with a 1% solution of Decon 90 followed by deionized water.

2.3. Bind-elute experiments combined with DSC analysis

Before buffer exchange to the experiment buffer containing 800 mM ammonium sulfate, a DSC thermogram of the model antibody in 20 mM phosphate buffer pH 7.3 was measured. Afterwards, antibody and stationary phase both in the experiment buffer containing 800 mM ammonium sulfate were incubated under end-over-end shaking for 4 h. After this incubation half of the sample was transferred into the sample cell of the calorimeter and a thermogram of the antibody bound to the stationary phase was measured. The other half of the sample was centrifuged in an Eppendorf 5415 R Benchtop Centrifuge, the supernatant liquid was removed and an equal volume of 20 mM phosphate buffer pH 7.3

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