Biophysical Chemistry 143 (2009) 70–78

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

A thermodynamic analysis of the binding interaction between polysorbate 20 and 80 with human serum albumins and immunoglobulins: A contribution to understand colloidal protein stabilisation

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ARTICLE INFO

Article history:
Received 26 January 2009
Received in revised form 3 April 2009
Accepted 8 April 2009
Available online 16 April 2009

Keywords:
ITC
DSC
Polysorbate
Albumin
Immunoglobulin
Antibody

ABSTRACT

The development of liquid therapeutic protein drugs imposes the presence of specific stabilisation agents to prevent protein degradation in order to reach shelf-lives of at least 2 years for drugs stored at 2–8 °C. Non-ionic detergents are used to avoid protein adsorption and the formation of protein aggregates. Depending on the protein and excipient (detergent) used the stabilisation effect is quite different and cannot be predicted up to now. One reason for this is the inadequate understanding of the principles that govern the stabilisation of proteins in the presence of detergents. One stabilisation mechanism discussed implicates a direct binding of detergent molecules to the hydrophobic surface area(s) of the protein in order to minimise protein–protein interactions and thus protein aggregation.

Therefore, the presented study considers the interaction and binding of polysorbate 20 and 80 to various human serum albumins and immunoglobulins of different subtypes. The interaction is analysed by means of isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). From ITC the binding constant is derived as well as the thermodynamic parameters. The thermal protein stability is obtained from DSC.

The results show that binding of the two detergents to human serum albumin is observed with binding constants of approximately $\approx 10^3$ M^{-1}, with 1–3 detergent molecules binding to the albumins. The exact polysorbate–albumin ratio depends on the used albumin fraction. The interaction of the detergent is also obvious from the DSC results, showing an increase of the denaturation temperature. However, the binding of the detergent to the three investigated immunoglobulins is quite low and negligible, thus showing that for immunoglobulins a direct and strong polysorbate binding to the protein is not the reason for the colloidal stabilisation effect of immunoglobulins in solution in the presence of polysorbate 20 or 80.

1. Introduction

The progresses achieved in the last 2 decades in genetics and biotechnology have encouraged the development of new therapeutic entities based on protein drugs [1,2]. Up to now protein therapeutics have to be applied via the parenteral route, with common protein concentrations varying between 0.1–25 mg/ml, depending on the used protein. Interferon therapeutics are presented as low concentration dosage forms, whereas antibodies can be formulated at much higher protein concentrations. For immunoglobulins the preferred route is a subcutaneous application. However, for these therapeutics the doses per application are quite high reaching protein concentration up to 100 mg/ml. For such protein dosage forms, beyond chemical stability, physical and colloidal stability of the protein in an aqueous solution becomes of high relevance [3–7]. The main physical degradation encountered is protein aggregation, which reduces bioactivity and product quality [8,9]. Furthermore, such protein aggregates are potentially immunogenic [10].

An important aspect to retain the biological activity of proteins is to maintain the protein structure in a specific, three-dimensional conformation [11]. This conformation, which can be investigated using different spectroscopic techniques [12–14], is only marginally stable with the free energy for the unfolded to folded, native state $\Delta G_{\mu\text{-f}}$ lying between −30 and −80 $kJ \cdot mol^{-1}$. Thus, fairly small perturbations in the secondary and tertiary proteins structure can induce a strong loss of biological activity. Changes of the secondary structure are also known to be one cause for the formation of protein aggregates [13]. A number of process steps can injure the three-dimensional protein structure like: sterile filtration, adsorption to interfaces, agitation, shear stress, freezing and thawing, drying via lyophilisation or spray drying, just to mention a few possible steps in the production of biopharmaceuticals [15,16].

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doi:10.1016/j.bpc.2009.04.004

To stabilise proteins in aqueous solutions, various excipients are used to increase the conformational stability against chemical and physical degradation [17–22]. Especially for the prevention of physical degradation, surface active agents like detergents are often necessary [17,18]. Therefore, a number of biophysical techniques are used to investigate and understand protein–excipient interactions [6,23–30]. Detergents are amphiphilic in nature, containing a hydrophilic head group and a hydrophobic part, and this dual property causes detergents to adopt a specific orientation at interfaces and in aqueous solutions.

In the literature a number of various mechanisms are discussed by which detergents may prevent protein damages and increase colloidal stability. Timasheff et al. [31,32] have developed a theory explaining the protein stabilisation effect, induced by the presence of excipients, being related to a preferential exclusion mechanism. Another theory considers the amphiphile nature of detergents. This implies that detergents are arranged and ordered at interfaces affecting the kinetics and thermodynamics of protein interfaces. For systems containing e.g. water/air interfaces the detergent molecules equilibrate between the aqueous bulk phase and the interface [31]. Thus, accumulation of detergent molecules at the water/air interface is observed inducing the formation of a detergent film with the polar head group of the detergent pointing in the aqueous bulk phase and the protein molecules remaining in the bulk phase.

Some proteins show a more or less pronounced surface activity, with its driving force for adsorption being a reduction of the entropy of water molecules that are ordered around the protein domains, especially arranged around the hydrophobic protein patches, when the protein is in an aqueous phase. Due to the fact that proteins show a certain surface activity, there will be a competition between protein and detergent molecules with regards to the accumulation at the water/air interface (co-adsorption), thus reducing the amount of proteins at the water/air interface. This is beneficial, because it is known that a number of proteins unfold irreversibly when exposed to the water/air interface.

Roth et al. [33] have shown that the detergent polysorbate 20 is able to displace β-lactoglobulin molecules from the water/air interface, and as a consequence, the protein concentration at the water/air interface is strongly reduced. This effect may be beneficial for the stabilisation of the protein in solution.

Surface adsorption is also relevant for other interfaces, as an example glass, tubing materials etc. As predicted by the Gibbs adsorption isotherm, the adsorption of detergents to interfaces will lower the surface tension. Considering a co-adsorption of detergent and protein molecules at an interface the protein driving force for surface adsorption will be lowered.

The protection effect related to the surface activity of the compounds in solution depends on the nature of the protein and detergent, is concentration-dependent and depends strongly on the CMC (critical micellar concentration) of the detergent.

Another mechanism attributes to certain excipients a “chemical chaperon” activity, aiding the refolding of protein, shifting the equilibrium to the native protein state and with this increasing protein stability [18].

Also changes of the protein hydration properties in the presence of excipients have been considered with regard to stability [34–36].

A further mechanism, often presented, considers a direct interaction and binding of the detergent with the protein [30,37–39]. It is discussed that such a binding is especially observed for proteins having hydrophobic amino acid residues close to the water interface. As a consequence, the binding of detergent molecules to such hydrophobic protein area (hydrophobic patches) will protect these against protein aggregation, because the detergent–protein complex becomes more “hydrophilic” compared to the pure protein. With this the protein–detergent complex becomes less surface active and will not so strongly compete with water/air interfaces. Bam et al. [38] have presented a study on the interaction of polysorbate 20 with a recombinant human growth hormone (rhGH) with regards to the ability of the detergent to prevent agitation-induced protein denaturation. The outcome of this investigation was that polysorbate 20 binding to the protein is driven by hydrophobic interactions and the presence of a polysorbate 20 to rhGH molar ratios >4 effectively inhibits protein aggregation, which was induced by agitation. A similar study was presented by Chou et al. [40], who investigated the effects of polysorbate 20 and 80 on the stability of albutropin during agitation. They found that polysorbate 20 and 80 had saturable binding to albutropin with a molar binding stoichiometry of 10:1and 9:1 (detergent: protein), respectively.

According to theses studies the protein stabilisation effect depends on the correct protein to detergent ratio.

However, Wang et al. [41] have recently shown that the detergent protection effect can be overbalanced by an increase of protein chemical instability. Protection against physical stress was obtained in the presence of polysorbate 80, however, chemical degradations of the protein increased in the presence of the detergent.

In a number of cases there is a detergent-dependent concentration optimum needed in order to stabilise the protein in solution [9]. Below this optimum protein aggregation is observed, at the optimum aggregation inhibition is induced, and with increasing the detergent concentration protein aggregation is again detected. These effects depend on the properties of the protein and detergent and up to now cannot be predicted.

The presented study deals with the interaction of polysorbate 20 and 80 to two classes of proteins: human serum albumin (HSA) and immunoglobulins (IgG). Two qualities of HSA were investigated; the usually available HSA containing a certain amount of fatty acid and a HSA fraction free of fatty acids (HSA-FAF). Human serum albumins were used, due to its known capacity to bind to fatty acids.

Three different immunoglobulins were investigated of the subtype IgG1, IgG2 and IgG4. The interaction and binding of the detergent to the proteins was analysed by means of isothermal titration calorimetry (ITC) and the conformational thermal stability analysed by differential scanning calorimetry (DSC).

It is discussed in the literature that hydrophobic protein patches (corresponding to hydrophobic areas or regions at the surface of the protein) are nucleation contacts inducing the formation of protein aggregates. The intention of the presented study was to verify the hypothesis whether detergent molecules like polysorbates interact with hydrophobic protein patches, and thus cover these patches making the patches more hydrophilic. It is believed that covering hydrophobic protein patches with detergent molecules reduces protein–protein interactions and thus reduces the propensity for the formation of protein aggregates. Therefore, the aim of the study was to distinguish whether there is a direct interaction and binding of detergent molecules to proteins and to determine the protein to detergent ratio for such a binding. Knowing the exact binding stoichiometry would allow a rational choice of detergent concentration in order to protect the protein from physical stress, and thus increase the colloidal stability of liquid protein solutions. Understanding the stabilisation mechanism would lead to a rational formulation design of a pharmaceutical product.

2. Experimental

2.1. Materials

The immunoglobulins were produced by mammalian cell culture technology. The purity of the proteins was determined by size exclusion chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis [42].

The monomer content of all IgG1 samples was >99% (based on high performance size exclusion chromatography [42]). The concentration

of the IgG sample solutions were determined by UV-measurement at 279 nm using an absorption of 1.32 for a 1 mg/mL solution (path length $d = 1$ cm).

Human serum albumin was purchased from Sigma-Aldrich GmbH (Munich, Germany). Two qualities were used. Albumin from human serum (99% pure based on agarose gel electrophoresis) denoted in this study as HSA and a human albumin (≈99%), essentially fatty acid (≈0.005%) and globulin free denoted as HSA-FAF (HSA fatty acid free). The concentration of the albumins was determined spectrophotometrically by UV-spectroscopy using the absorption at a wavelength of 280 nm ($\varepsilon_{280} = 0.51$ OD/(mg/ml)). Buffer and inorganic salts (Sigma-Aldrich Chemie GmbH, Munich) were of analytical grade. The detergents polysorbate 20 (polyoxyethylen-sorbitan-monolaurat, $C_{58}H_{114}O_{26}$) and 80 (polyoxyethylen-sorbitan-monooleate, $C_{64}H_{124}O_{26}$) were obtained from Croda (Edison, NJ).

2.2. Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed with a cap-DSC calorimeter from MicroCal™, Inc. (Northampton, MA). The protein concentration was set to 4 mg/ml. The scan rate was 1 K/min. Calorimetric traces were recorded from 10–95 °C. Only the first heating scan is shown in the graphs of this study, because of total denaturation of the immunoglobulin samples after heating above 95 °C. However, just the temperature intervals where effects are observed are represented in the figures. A reference scan was run under identical DSC set up conditions and subtracted from each sample scan. The experiments were repeated three times to show reproducibility of the results. The heat capacity curves were analysed using the ORIGIN® software. The denaturation temperature is determined as the maximum of the heat capacity curve, which is obtained with a precision of ±0.1 K.

2.2.1. Isothermal titration calorimetry

The calorimetric titration experiments were performed using a VP-ITC from MicroCal™, Inc. (Northampton, MA). The titration experiments were carried in the temperature range from 5 to 55 °C. Prior to each titration experiment, the sample cell and the syringe were rinsed with freshly distilled water and then shortly with buffer. The reference cell was filled with degassed buffer. The 1.4 ml reaction cell was loaded with the protein solution, whereas the injection syringe (nominal volume 250 µl) was filled with the detergent solution.

The ITC instrument was equilibrated at a temperature of 5 °C below the experimental temperature. The initial delay time was 60 s. The reference power and the filter were set to 10 µcal/s (corresponding to 42 µJ/s) and 2 s, respectively.

A representative titration experiment consisted of 25 injections of 10 µl with an injection speed of 0.5 µl/s. The syringe is filled with a detergent solution at concentrations of 10 mM and the protein concentration in the cell was between 0.15 and 0.24 mM. The buffer composition was equal for the detergent and protein solution. The time interval between two consecutive injections was set in order to allow the heat signal to return to the baseline. During the experiments, the sample solution was continuously stirred at 300 rpm by the rotating paddle attached to the end of the syringe needle.

The titration curves were analyzed using the ORIGIN® software provided with the calorimeter. A binding model with identical and independent binding sites was used to fit the data. Data of the first injection were discarded due to inaccurate volume and concentration because of a possible dilution of the protein solution in the syringe needle during thermal equilibration. Each experiment was repeated 3 times under the same conditions in order to determine the precision of the results and to ensure their reproducibility. The thermodynamic parameters and errors were then calculated and averaged.

3. Results and discussion

Two different polysorbate detergents were studied: polysorbate 20 and 80. Polysorbate 20 is a polyoxyethylensorbitan-monolaureate and polysorbate 80 the corresponding monooleate. The critical micellar range of polysorbates lies between 5–50 µM, depending on the temperature and solution conditions. Both detergents show acceptable toxicity in order to be used in injectables. A number of commercially available parenteral products contain polysorbate 20 and 80 like Avastin®, Lucentis®, Campath® or Humira®. The effect of these detergents was investigated in concentration ranges of pharmaceutical relevance (0–2 mM). Higher detergent concentrations were not considered.

3.1. Interaction of polysorbate 20 and 80 with human serum albumin (HSA)

A typical titration experiment consists of a step by step titration of detergent to a protein solution at a constant temperature. Each injection induces an exothermic reaction (Fig. 1). The measured reaction heat is the sum of dilution, demicellisation and reaction enthalpy. The dilution and demicellisation enthalpy is obtained from a separate experiment by titrating detergent solution into buffer (of the same composition). In Fig. 1, a reference titration experiment is exemplarily shown. For the first injection of polysorbate 20 to HSA the reaction heat is about -25 kJ·mol^{-1} (15 °C) and during the titration

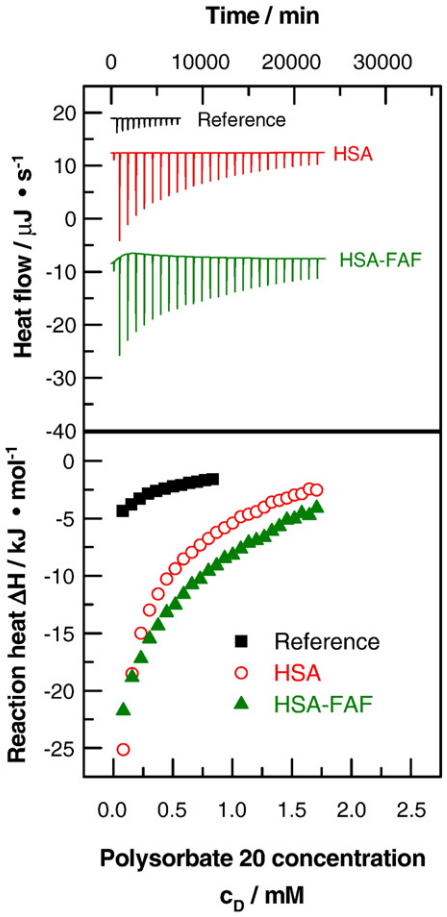

Fig. 1. Top: Experimental power flow signals of the interaction of polysorbate 20 (10 mM) with human serum albumin (HSA) and human serum albumin fatty acid free (HSA-FAF) at 25 °C. The protein concentration is 0.24 mM. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl. The corresponding reference experiment, titration of polysorbate 20 into buffer, is also shown. Bottom: Binding isotherms representing the reaction enthalpies as determined by peak integration.

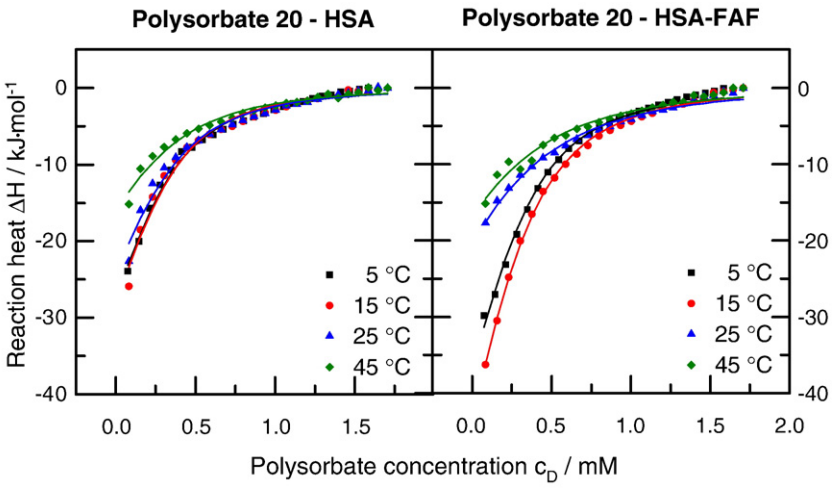

Fig. 2. Binding isotherms of polysorbate 20 to human serum albumin (HSA) and human serum albumin fatty acid free (HSA-FAF) between 5 and 45 °C. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl.

experiment the reaction heat decreases. At the end of the titration experiment the observed reaction heats are mainly due to dilution and demicellisation. Similar results are observed for the titration of polysorbate 80 (data not shown).

The temperature-dependences of the binding isotherms upon the interaction of polysorbate 20 to HSA are shown in Fig. 2. The experiments were performed between 5 and 45 °C, at temperature below the onset of protein denaturation. At higher temperatures an increase of the reaction enthalpy was observed due to unspecific binding of the detergent to partially denaturated (by heat) protein (data not shown).

The results presented in Fig. 2 show, with increasing the temperature, the reaction enthalpy being reduced and becoming less negative. Such a temperature induced behaviour shows that entropic effects reduce the interaction of the detergent to the protein. The detergent to protein molar ratio is 1–2. However, higher binding ratio has been described for non-ionic detergents. For example, the non-ionic detergent Triton X-100 binds to bovine serum albumin with a maximum of four detergent molecules per protein [37].

Using a binding model considering just one set of sites [43], the binding constant K_a as well as the thermodynamic parameters ΔH^0 (change in enthalpy) is obtained. ΔG^0 (change in free energy) is calculated based on K_a according to $\Delta G^0 = -R \cdot T \cdot \ln K_a$, with R the gas constant and T the temperature. The change in entropy (ΔS^0) is calculated using the Gibbs–Helmholtz relationship. At 25 °C these parameters for the interaction of polysorbate 20 with HSA are: K_a (25 °C) = 1600 ± 110 M^{-1}, $\Delta H^0 = -54 \pm 2$ kJ·mol^{-1}, $T \cdot \Delta S^0 = -34 \pm 2$ kJ·mol^{-1}, and $\Delta G^0 = -18 \pm 0.2$ kJ·mol^{-1} (Fig. 3). A favourable negative binding enthalpy is due to an increase of van der Waals interactions and/or the formation of hydrogen bonds between the protein and the detergent upon binding. This is realised between the hydroxyl and/or polyethylene groups of polysorbate and the amino and carboxyl groups of the protein amino acids. The favourable enthalpic contribution of the polysorbate–HSA interaction is counter balanced by an unfavourable entropy term, leading to a smaller free binding energy of approximately −20 kJ·mol^{-1}. The interaction of polysorbate 80 with HSA is weaker, but similar in nature (data not shown). This is in accordance to the study presented by Chou et al. [40]. The weaker binding of polysorbate 80 to HSA may be due to the bulkier fatty acid (oleate chain) which reduces the interaction of the detergent with the protein.

Comparing the polysorbate 20 binding interaction of HSA with the corresponding BSA (bovine serum albumin) interaction one observes a very similar pattern (Fig. 3). The binding constant of BSA to polysorbate 20 is larger with K_a(25 °C) ≈ 11,000 M^{-1}.

The temperature dependency of the thermodynamic binding parameters are summarised in Fig. 4. The change in free energy ΔG^0 is negative with approximately −20 kJ·mol^{-1} and basically temperature independent. The reaction enthalpy slightly decreases with increasing temperature.

These results are consistent with the binding reaction of polysorbates with BSA and with the fluorescence results presented by Liu et al. [7].

The heat capacity curve of HSA in 25 mM sodium citrate at pH 6 with 115 mM NaCl shows two maxima located at 74.5 and 80.5 °C

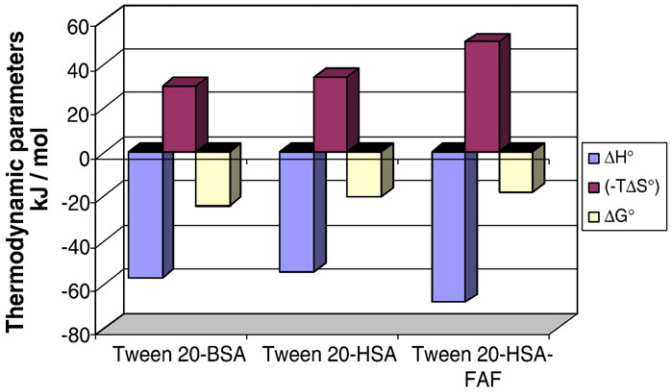

Fig. 3. Thermodynamic parameters (25 °C) of the interaction of polysorbate 20 with bovine serum albumin (BSA), human serum albumin (HSA) and human serum albumin fatty acid free (HSA-FAF). Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl. ΔH^0: change in enthalpy, T: temperature, ΔS^0: change in entropy, ΔG^0: change in free energy.

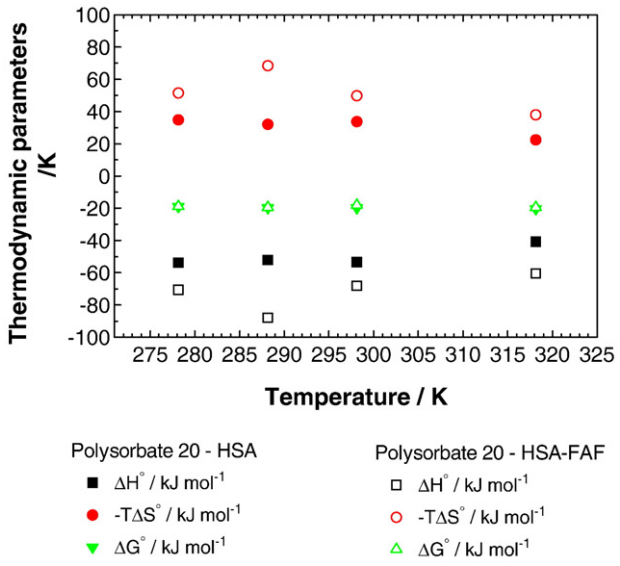

Fig. 4. Temperature dependency of the thermodynamic parameters ΔH^0 (change in enthalpy), ΔS^0 (change in entropy), and ΔG^0 (change in free enthalpy) for the interaction of polysorbate 20 to human serum albumin (HSA) and human serum albumin fatty acid free (HSA-FAF). Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl.

(Hoffmann et al., unpublished results). The shape of the HSA thermogram as obtained from DSC (differential scanning calorimetry) is in accordance to the data published by Michnik et al. [44]. The presence of polysorbates has a strong impact on the heat capacity curve. Small amounts of polysorbate induce the disappearance of the two maxima and just one maximum located around ≈77 °C is detected and with increasing detergent concentration a shift of the heat capacity is observed accompanied with an increase of the denaturation enthalpy (see below). This effect is observed for both polysorbates and the effects are similar (data not shown). The DSC results indicate that the presence of polysorbate interact with HSA which is consistent with the ITC results.

3.2. Interaction of polysorbate 20 and 80 with human serum albumin fatty acid free (HSA-FAF)

The reaction enthalpies for the titration of polysorbate 20 and 80 to fatty acid free HSA (HSA-FAF) are exothermic (Fig. 1) as observed for the other albumins. With increasing the temperature the reaction enthalpy decreases (Fig. 2). Using the one set of sites binding model (see above) the binding parameters of polysorbate 20 to HSA-FAF at 25 °C are: $K_a(25\ °C) = 3000 \pm 290\ M^{-1}$, $\Delta H^0 = -68 \pm 3\ kJ \cdot mol^{-1}$, $T \cdot \Delta S^0 = -50 \pm 2\ kJ \cdot mol^{-1}$, and $\Delta G^0 = -20 \pm 0.2\ kJ \cdot mol^{-1}$ (Fig. 3). This shows that the binding constant of the detergent to HSA-FAF is slightly larger, compared to the HSA-system.

Comparing the thermodynamic signatures of the three investigated albumins (Fig. 3) one can deduce that the binding mechanism of polysorbate to the albumins is very similar. The free energy ΔG^0 is similar for the three systems. The favourable and larger reaction enthalpy ΔH is counter-balanced by an unfavourable more negative ΔS. This is also observed by analysing the temperature dependency of polysorbate 20 interaction with HSA-FAF (Fig. 4).

As described before, the polysorbate interaction with the albumin is also obvious from the analyses of the heat capacity curves. In contrast to the heat capacity curve of HSA, which shows two maxima, the heat capacity curve of HSA-FAF is represented by just one maximum (Fig. 5). The two transitions observed in HSA are likely to be due to albumin fraction composed of fatty acid-bound albumin and fatty acid free albumin. The presence of the naturally occurring fatty acids in the HSA albumin fraction stabilise the albumin; the maxima of the heat capacity curve are shifted to higher temperatures for the HSA compound compared to its fatty acid free counterpart (HSA-FAF). This matches with the results published by Michnik et al. [44].

The maximum of the denaturation temperature T_m of HSA-FAF is located at 69.3 °C with a denaturation enthalpy ΔH_m of 1025 $kJ \cdot mol^{-1}$. The presence of polysorbate 20 (Fig. 5, left) and polysorbate 80 (Fig. 5, right) induces T_m-shifts to higher temperature. In the presence of a detergent concentration of 2 mM, the protein T_m is increased for both detergents by 2.3 K, indicating an interaction of the detergent molecules with HSA-FAF. An increase in ΔH_m is also observed. In the presence of 2 mM detergent the denaturation enthalpy increases by approximately 25%, for both polysorbates.

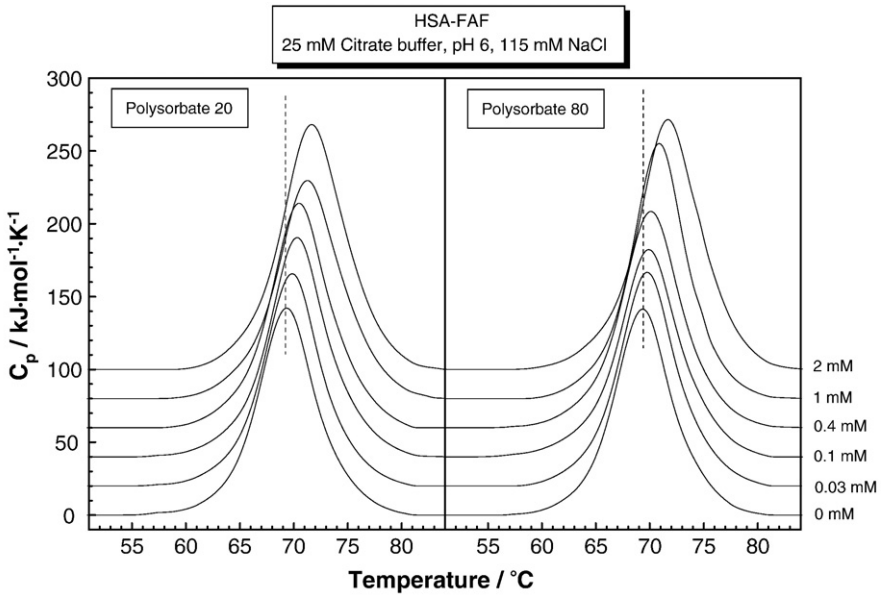

Fig. 5. Heat capacity curves (C_p) of human serum albumin fatty acid free (HSA-FAF) in the presence of different concentration (0, 0.03, 0.1, 0.4, 1, and 2 mM) of polysorbate 20 (left) and polysorbate 80 (right). Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl.

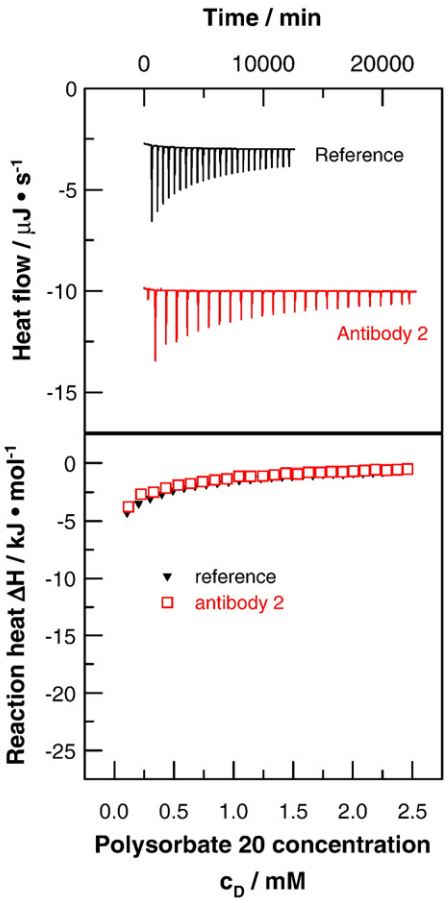

Fig. 6. Top: Experimental power flow signals of the interaction of polysorbate 20 (10 mM) with an immunoglobulin (ab 2) at 15 °C. The protein concentration is 0.17 mM. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl. The corresponding reference experiments, titration of polysorbate 20 into buffer, is also shown. Bottom: Binding isotherms representing the reaction enthalpies as determined by peak integration.

3.3. Interaction of polysorbate 20 and 80 with immunoglobulins (IgG)

The binding of polysorbate 20 and 80 to three different immunoglobulins of different subtypes IgG1 (ab 1), IgG2 (ab 2) and IgG4 (ab 3) are investigated. A typical ITC experiment at 25 °C is shown in Fig. 6. Comparing the polysorbate 20 binding reaction to the immunoglobulin with the reference experiment, it becomes apparent that the binding reaction is extremely low up to being negligible. The temperature dependency of the binding of ab 2 with both detergents is shown in Fig. 7, illustrating that with temperature increase the reaction enthalpy is even reduced. This corresponds to the results observed for the albumins. Furthermore, the reaction enthalpies of polysorbate 80 with the immunoglobulin ab 2 is much lower compared to the reaction enthalpies observed for the corresponding polysorbate 20 system. This is also the case for the other two immunoglobulins (data not shown) and corresponds to the results obtained for the two human serum albumins. The reaction enthalpy of polysorbate 80 with human albumins and immunoglobulins is lower compared to polysorbate 20. A similar effect is observed for lysozyme [52]. For bovine serum albumin this trend is also experienced, however the difference between the reaction enthalpies of the two detergents is smaller.

The "strongest" interaction of the detergents with the three investigated immunoglobulins is found for ab 1, which is an IgG1 (see Fig. 9). Whether this can be attributed to the immunoglobulin subtype is actually unclear. In order to elucidate if polysorbates show a slightly stronger interaction to IgG1 immunoglobulins, additional immunoglobulins of the same subtype have to be investigated. However the binding constants are extremely small with values in the order of 10^2–10^3 M^{-1}. Compared to the interaction of immunoglobulins with protein A, where binding constants of 10^7–10^8 M^{-1} are obtained [43], the detergent binding to immunoglobulins is quite negligible.

The heat capacity curve of an immunoglobulin (ab 1) in the presence of polysorbate 20 is exemplarily shown in Fig. 8. The heat capacity curve of the immunoglobulin is represented by two endotherms which is the result of the different thermal stability of the Fab and Fc parts of the immunoglobulin [45]. The exact position of the denaturation temperatures as well as the enthalpies varies depending on the environmental conditions and immunoglobulin subtype [14,46]. The overall denaturation enthalpy for immunoglobulin varies between 2000 and 5000 kJ·mol^{-1}, depending on the used framework [45,47]. Fig. 8 represents the thermogram of an IgG1 (ab 1), with the two melting temperatures located at 74.1 and 82.7 °C. The overall denaturation enthalpy is 4125 kJ·mol^{-1} (25 mM sodium citrate, pH 6, 115 mM NaCl), which is in accordance with published data [45]. The analysis of the heat capacity curve shows that the presence of

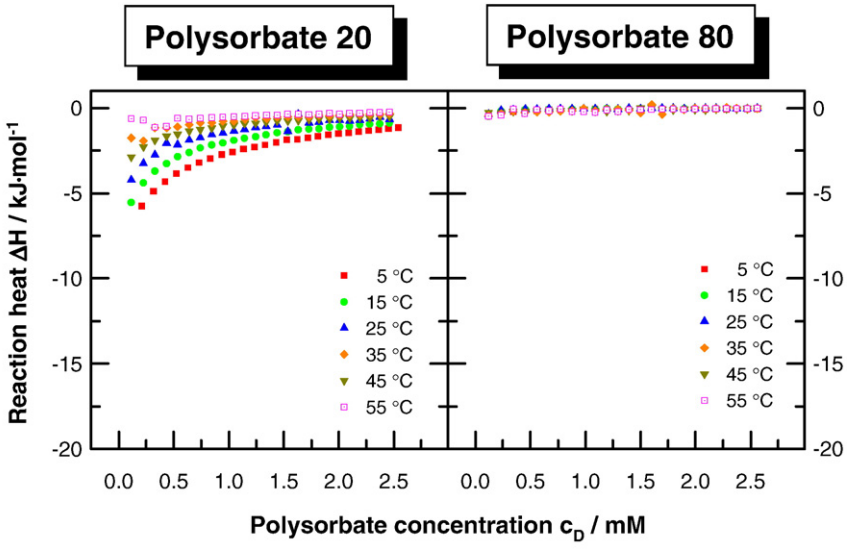

Fig. 7. Binding isotherms of polysorbate 20 (left) and polysorbate 80 (right) to an immunoglobulin (ab 2) between 5 and 55 °C. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl.

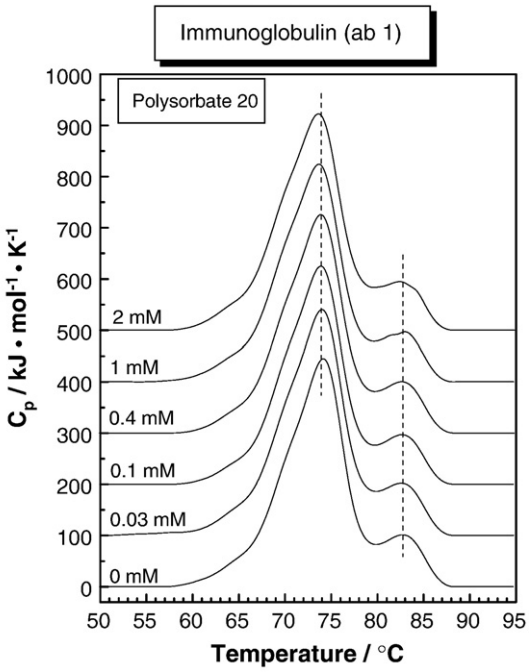

Fig. 8. Heat capacity curves (C_p) of an immunoglobulin (ab 1) in the presence of different concentration (0, 0.03, 0.1, 0.4, 1, 2 mM) of polysorbate 20. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl.

the detergent with regards to the shape and thermodynamic parameters of the immunoglobulin is quite negligible. No relevant changes of the denaturation enthalpy are observed. In the presence of 2 mM polysorbate 20 a slight T_m shift of ≈0.3 K to higher temperature, is detected. The presented results indicate a very weak detergent–immunoglobulin interaction, compared to the much stronger interactions described for the human serum albumin–polysorbate systems (see Fig. 5). These results are consistent with the ITC results.

4. Conclusions

The underlying study is focussed on the interaction of the non-ionic detergents polysorbate 20 and 80 with human serum albumin and immunoglobulins. The interaction of ionic detergent like SDS (sodium dodecyl sulphate) is in general much stronger inducing the denaturation of the protein component. Therefore, in pharmaceutical products non-ionic detergents are used. Furthermore, non-ionic detergents like polysorbate 20 and 80 are well tolerated as excipients used in injectables [48–50].

To understand the polysorbate–human serum albumin interaction one has to consider the protein structure.

Human serum albumin is a heart-shaped, monomeric protein composed of 585 amino acids (molecular weight = 66.4 kDa). The secondary structure of HSA is mainly composed of alpha-helices (ca. 67%), nearly entirely lacking beta-sheets. Human serum albumin is organised into three homologous domains, which are labelled I, II and III. Each of these domains is subdivided into two sub-domains termed A and B. The latter share common structure elements [53]. The number of disulphide bonds is 17. They are exclusively intra-sub-domain disulphide bonds, which is the reason for the high thermostability of human serum albumin (see Fig. 5. and discussion below). The solubility of human serum albumin is quite high; it is the major protein component of blood plasma with a concentration of 0.6 mM. As described above, the primary role of human serum albumin is the transport of fatty acids. Under normal physiological conditions 0.1–2 mol fatty acids bind to 1 mol human serum albumin. However, larger molar ratio of fatty acid to human serum albumin of 6:1 were reported, especially in the peripheral vasculature during fasting or extreme exercises, or under pathological conditions such as diabetes, liver and cardiovascular diseases [54–59].

Up to seven different binding sites have been reported in the literature with varying affinities for fatty acids. Dissociation constants in the μM up to nM range have been determined [53,60]. From the crystal structure of fatty acid–human serum albumin complexes five of the seven binding sites are described as high affinity binding sites, because they possess basic or polar amino acid side chains that may interact closely with the carboxyl group of bound fatty acid. For a strong interaction of the fatty acid with the albumin a hydrophobic binding pocket is required in addition to electrostatic interactions of the carboxyl group with corresponding amino acids. Simard et al. [56] point out that just hydrophobic interactions alone are not sufficient enough to stabilise the binding of fatty acids.

The fatty acid to human serum albumin binding depends on a number of factors like acyl chain length, degree of saturation of the hydrocarbon chain, degree of protonation of the fatty acid etc, just to name a few. This has been described in the literature. For more detail see Bhattacharya et al. [59], Petitpas et al. [60], Zunszain et al. [61], and Sugio et al. [62].

Thus, only one requirement for a strong interaction of polysorbates with HSA is given; namely a hydrophobic hydrocarbon chain–protein interaction is expected, which maybe is enhanced by hydrogen bonds between the ethylene oxide units of the detergent as acceptor sites with H-bond donor sites of the protein.

Our calorimetric results show that binding of the detergent molecules to human serum albumins is observed, with low binding constants of $\approx 10^3$ M^{-1}. 1–2 detergent molecules bind to HSA, whereas 2–3 molecules bind to HSA fatty acid free (HSA-FAF). Detergent interaction is expected, because human serum albumin is a fatty acid binding protein. However, considering the information available form x-ray investigations for fatty acid–human serum albumin complexes, the low binding constants observed for polysorbates are due to the fact that the acyl chain of the detergent just barely fits the hydrophobic binding pocket of the albumin.

Using the energetic signatures (ΔG^0, ΔH^0, ΔS^0) it is possible to determine the interaction mechanism. The energetic source of polysorbates interacting with human and bovine serum albumins is characterised by a dominant negative enthalpy indicating the formation of large numbers of favourable bond contacts and/or van der Waals interactions upon binding. This is however counterbalanced by unfavourable conformational changes in either or both interaction partners, expressed in the negative entropy parameter. Thus low binding constants are observed.

The interaction of polysorbate 20 with the proteins is slightly stronger compared to polysorbate 80. Using a human serum albumin protein fraction where the fatty acid components were removed, a slightly stronger detergent binding constant compared to fatty acid containing human serum albumin is observed. This is in accordance with the fatty acid binding role of serum albumins, however, the effects are small, which leads to the conclusion that the serum albumin binding sites do not really fit to the fatty acid of the polysorbate. It is likely that this is due to steric hindrance effects of the detergent fatty acid upon binding to the albumins.

Although the colloidal stabilisation of immunoglobulins against physical stress is favoured by the presence of polysorbate 20 and 80 [51], the presented results show that the protective mechanism is not related to a specific detergent–immunoglobulin binding. This is evident from our results, which have shown for three immunoglobulin subtypes (IgG1, IgG2 and IgG4) that the binding of the detergent is quite negligible. Fig. 9 summarises the binding isotherms of the five investigated proteins for the interaction with polysorbate 20 at 25 °C. A binding of polysorbate 20 to human serum albumins is obvious, but negligible for the immunoglobulins. These results are confirmed by fluorescence spectroscopic measurements [52].

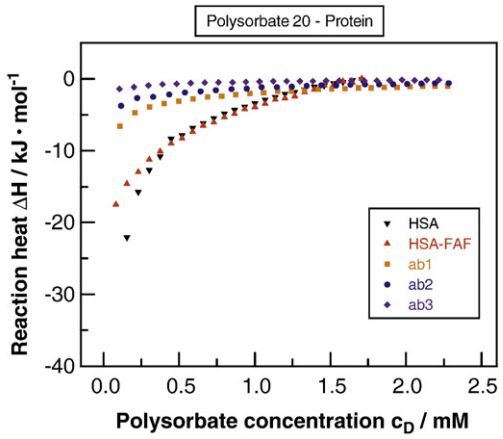

Fig. 9. Comparison of the binding isotherms of polysorbate 20 to various proteins: human serum albumin (HSA) and human serum albumin fatty acid free (HSA-FAF) and three immunoglobulins denoted as ab 1, ab 2, and ab 3 (25 °C). Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl.

Compared to human serum albumin, the structure of immunoglobulins is quite different. All immunoglobulins are Y-shaped with a four chain structure as their basic unit. The main secondary structure element of immunoglobulins is beta-sheet structure with approx. 48%, whereas the amount of alpha-helical structure is below 11% [12]. They are formed of two identical light chains (LC) of 23 kDa and two identical heavy chains (HC) of 50–70 kDa. The HC and LC are held together by inter-chain bonds, which vary among the IgG subtypes and by non-covalent interactions. The HC and the LC chains are further subdivided into two regions based on variability in the amino acid sequences. Glycosilation is found at the so-called CH2 domain. The difference with the various human IgG subclasses lies in a slight change of the amino acid sequence. Most of the differences are clustered in the hinge region and therefore, give rise to differing patterns of interchain disulphide bonds between the IgG subtypes. This is especially pronounced for IgG3, which shows an elongated hinge region. For the other three subtypes, used in this study, this is not so relevant [63]. For the three investigated immunoglobulins, the presence of highly hydrophobic patches exposed to the solvent is not likely, because the detergent binding to the proteins is basically not observed.

Thus, as a consequence for the development of immunoglobulin biopharmaceuticals the presented results suggest that there is no specific detergent to protein (immunoglobulin) ratio necessary to stabilise liquid protein formulations against the formation of protein aggregates.

Further studies have to show whether this effect can be generalised, and additional immunoglobulins have to be investigated.

Due to the fact that polysorbate–immunoglobulin binding is negligible other mechanisms seem to play a role for the colloidal stabilisation effect. This will be addressed in following studies.

Acknowledgements

We thank Inge Miller and Heidrun Schott for continuous technical support, and Werner Kliche for helpful discussion. Stefan Bassarab is acknowledged for his strong interest and support.

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