Eur Biophys J (2009) 38:557–568
DOI 10.1007/s00249-009-0404-6

ORIGINAL PAPER

Insights into protein–polysorbate interactions analysed by means of isothermal titration and differential scanning calorimetry

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Received: 30 October 2008 / Revised: 1 January 2009 / Accepted: 2 January 2009 / Published online: 3 February 2009

Abstract Therapeutic proteins formulated as liquid solutions at high protein concentration are very sensitive to chemical and physical degradation. Especially avoiding the formation of protein aggregates is very crucial for product quality. In order to stabilize the colloidal properties of protein therapeutics various excipient are used. Especially the detergents polysorbate 20 and 80 are common. However, the mechanism upon which the detergents protect the protein from aggregation is not really known. The present study investigates the interaction of polysorbate 20 and 80 with different proteins: lysozyme, bovine serum albumin (BSA) and an immunoglobulin. The interaction and binding of the detergents to the proteins is investigated by isothermal titration calorimetry (ITC). From ITC the thermodynamic parameters (ΔH: change in enthalpy, ΔS: entropy and ΔG: free energy) upon binding are derived as well as the binding constant K_a. The thermal stability of the proteins in the presence of the detergent is assessed by differential scanning calorimetry (DSC). The results show that both detergents bind to BSA with K_a between 8 and $12 \times 10^3\ M^{-1}$ with ΔH −50 to −60 kJ/mol (25°C). One to two detergent molecules bind to BSA. The presence of both detergents induces a weak stabilisation of the thermal denaturation properties of BSA. However, the interaction of polysorbate 20 and 80 with lysozyme and the immunoglobulin is quite negligible. The presence of the detergents up to a concentration of 2 mM has no impact on the heat capacity curve neither a destabilisation nor a stabilisation of the native conformation is observed.

Keywords Calorimetry · Immunoglobulin · BSA · Lysozyme · Tween · Polysorbate

Abbreviations

ITC	Isothermal titration calorimetry
DSC	Differential scanning calorimetry
IgG	Immunoglobulin
BSA	Bovine serum albumin
Lys	Lysozyme

Introduction

In the last years the knowledge of the production of protein therapeutics (e.g. immunoglobulins) using biotechnological processes has increased dramatically (Werner 2004). Nowadays, monoclonal antibodies can be produced using mammalian cell culture (CHO: Chinese hamster ovary cells) up to titers of $4\ g\ l^{-1}$ in a 11-day fed-batch process (Bergeman et al. 2007).

Although liquid solutions of protein therapeutics are very sensitive to a number of degradation pathways, there is a high interest for the development of liquid formulations (Remmele et al. 2005, 2006; Kerwin and Remmele 2007; Kanai et al. 2008). The interests lie in the development of patient and medical friendly dosage forms as well as in the production of cost effective drugs. The amount of protein drug delivered per application, especially for antibodies, can be quite high. Amounts of a few hundreds of mg of antibody drug are common. Furthermore, for patient safety

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and convenience antibodies should be delivered via the subcutaneous route. This implicates the development of protein formulations up to concentration of 100 mg/ml. However, for such high concentrated liquid formulations the avoidance of protein aggregation is a challenge (Cromwell et al. 2006; Shire et al. 2004; Wang 2005; Garidel and Bassarab 2008).

Protein aggregation can be observed under a wide variety of process conditions. The rate and extent of protein aggregation depends on a number of factors like the physico–chemical property of the protein, solution conditions, protein concentration, and the presence of excipients. However, also process factors including sterile filtration, pumping, freeze/thaw cycles, product transportation (liquid drug products) may induce the formation of aggregates.

Therefore, a number of excipients are used and tested in order to increase the colloidal stability of the protein solution.

Various techniques are used to investigate protein–excipient interactions. These techniques include spectroscopic methods like fluorescence spectroscopy (Nielsen et al. 2005; Weichel et al. 2008; Garidel 2008; Kragh-Hansen et al. 2001; Garidel et al. 2008; Otzen et al. 2009), infrared spectroscopy (Lad et al. 2003; Garidel and Schott 2006), photometry (Sereikaite et al. 2005; Yonath and Blauer 1974), electron paramagnetic resonance (Bam et al. 1995), chromatography (Møller and le Maire 1993; LeBrun et al. 2009), scattering techniques (Santos et al. 2003; Valstar et al. 1999, 2000), calorimetry (Lad et al. 2003; Lund et al. 2006; Bagger et al. 2007), surface tension (Santos et al. 2003; Lad et al. 2003), surface pressure (Wu et al. 2006), nuclear magnetic resonance (Chen et al. 1995), equilibrium dialysis (Wasylewski and Kozik 1979; Kragh-Hansen et al. 2001; Cardamone et al. 1994; Reynolds and Gallagher 1970) analytical ultracentrifugation (Jones et al. 1999; Liu and Shire 2000) and computer simulation (Bond et al. 2005, 2006) just to mention a few methods.

One class of excipients used to prevent protein aggregation is non-ionic detergents. Among these polysorbate 20 and 80 are the most common excipients (Ashford and Landi 1966; Good and Selin 1979; Iourtov et al. 1999; Kamande et al. 2000; Jones et al. 2001; Hillgren et al. 2002; Wang 2005). These detergents are used for the development of parenteral products, because they have low toxicological properties and are well tolerated (Coors et al. 2005; see Final report on the safety assessment of polysorbates 20, 21, 40, 60, 61, 80, 81, and 85, 1984). Polysorbate 20 and 80 are found in different commercially available parenteral protein products. For examples, the humanised monoclonal antibody (IgG1) Avastin® contains 0.4 mg/ml polysorbate 20, Lucentis® contains 0.01% polysorbate 20. Polysorbate 80 is a formulation constituent of Campath® (IgG1) at a concentration of 0.1 mg/ml. Other biopharmaceutical products containing polysorbate 80 are Humira® (IgG1 k), Orthoclone® (IgG2a), Remicade® (murine monoclonal antibody), Rituxan® (IgG1 k), Eprex®/Erypo® (epoetin) (Hermeling et al. 2003, Wang et al. 2007).

Detergents like polysorbates are used to stabilise protein therapeutics, especially to minimise protein aggregation. Polysorbates are also used to stabilise emulsions and solid lipid particles (Dickinson et al. 1999; Dimitrova and Leal-Calderon 1999; Göppert and Müller 2003). However, the influence of these detergents with regards to the avoidance of protein aggregation can not be predicted. Depending on the nature of the protein, polysorbates may stabilise or destabilise the protein solution against the formation of aggregates. Furthermore, there is a detergent concentration dependent optimum for stabilisation. Below this optimum, protein aggregation is not fully reduced, above the optimum; aggregation is again observed (Wang 2005). Depending on the applied stress, the effect of the excipient is quite different. Wang et al. (2008) have recently investigated the effect of polysorbate 80 for the stabilisation of a recombinant human IL-2 (interleukin-2) mutein. They found that through shaking induced protein aggregates of IL-2 mutein, the formation of protein aggregates was significantly inhibited in the presence of polysorbate 80. On the other hand, the presence of polysorbate 80 strongly affected the stability of IL-2 mutein formulated as a liquid solution. During accelerated stability storage conditions at 40°C product oxidation as well as the formation of protein aggregates were observed for polysorbate 80 containing formulations. One reason for this could be due to traces of peroxide which were introduced with the polysorbate excipient. However, they concluded, that there is a dual effect of polysorbate 80 on IL-2 stability.

Up to now, the protective effects of detergents are not really understood. Various possible stabilisation mechanisms are discussed. It is described that the presence of certain excipients may influence the solution property of the protein and thus influences protein hydration. These ideas are summarised under the concept of "preferential hydration" and "preferential exclusion" (Timasheff 2002; Shimizu and Smith 2004). Another stabilisation mechanism considers the adsorption competition between detergent and protein molecules at the water/air interface in the sense that the detergent molecules repulse the protein molecules in the bulk phase limiting thus the amount of protein molecules exposed to the air interface. Thus the surface tension may play a role (Lin and Timasheff 1996). Similar considerations are discussed for protein interactions at other interfaces (e.g. glass or tubing interfaces) (Bhat and Timasheff 1992; Timasheff 1998). Furthermore, detergents are also believed to act as chaperones shifting the equilibrium of partially unfolded state to the native protein conformation (Bam et al. 1995). Another often discussed concept, considers a direct interaction of the detergent with the protein

molecule. It is believed that detergent molecules may interact and/or bind to exposed hydrophobic protein regions, thus covering and protecting hydrophobic sites that may be the cause for protein aggregation (stabilisation of hydrophobic patches) (Nishikido et al. 1982; Peyre et al. 2005; Bam et al. 1998; Xie and Timasheff 1997).

Despite the complexity of protein–detergent interactions, the following study will focus only on the last aspect mentioned above, namely the question whether a direct interaction and/or binding between the detergent polysorbate and proteins can be observed.

The presented study considers therefore the interaction and binding of polysorbate 20 and 80 to three different proteins: lysozyme (lys), bovine serum albumin (BSA) and an immunoglobulin (IgG). The proteins differ in their physico–chemical properties and size. For example, the molecular weights of the proteins differ: MW(Lys) = 14.6 KDa, MW(BSA) = 66.4 KDa and MW(IgG) = 150 KDa. The isoelectric point pI of the proteins are: pI(Lys) = 11, pI(BSA) = 4.5 and pI(IgG1) = 8 (Chiodi et al. 2005; Chaiyasut and Tsuda 2001).

The protein–detergent interaction was investigated by means of isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). ITC is a unique technique allowing the direct determination of the binding constant (K_a), binding stoichiometry (n), as well as the binding enthalpy (ΔH: change in enthalpy). The other thermodynamic parameters (ΔS: change in entropy, ΔG: change in free enthalpy) are calculated using elementary thermodynamic relations (Arouri et al. 2007). These parameters allow the understanding of the nature of protein–detergent interactions. Once a detergent interacts directly or even binds to a protein molecule, it may either stabilise or destabilise the native conformation of the protein depending on the detergent type, molar ratio, and solution conditions. The influence of the presence of detergent with regards to the thermally induced protein denaturation is analysed by DSC. From the heat capacity curve, the denaturation temperature (T_m) is defined as the maximum of the heat capacity curve. This parameter indicates, in a first assumption, whether the presence of the excipient may stabilise or destabilise the native protein structure. Information considering the enthalpy contributions are obtained by integrating the heat capacity curve (ΔH_{den}). The influence of the detergents was investigated for concentrations relevant for pharmaceutical formulations (0–2 mM). Higher detergent concentrations were not considered. The interaction between the proteins and the detergents was investigated in the temperature range between 5 and 55°C, thus below temperatures inducing thermal protein denaturation.

The aim of the study was to analyse whether polysorbate 20 and 80 interact and bind to the proteins at all, to determine the strength and stoichimetry of binding, and to study their impact on thermal stability of the proteins.

Materials and methods

Materials

Recombinant human immunoglobulin IgG sample was provided by Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany. The concentration of the IgG sample solutions were determined by UV-measurement at 279 nm using an absorbance of 1.32 for a 1 mg/mL solution (path length $d = 1$ cm). Purity was determined by size exclusion chromatography. The monomer content of all IgG samples was >99%.

Bovine serum albumin and lysozyme from chicken egg white were purchased from Sigma-Aldrich GmbH (Munich, Germany). Buffer and inorganic salts (Sigma-Aldrich Chemie GmbH, Munich) were of analytical grade. 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).

Methods

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 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 and 2 s, respectively.

A representative titration experiment consisted of 25 injections a 10 µl with an injection speed of 0.5 µl/s. 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 analysed 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 three times under the same conditions in order to determine the

accuracy of the results and to ensure their reproducibility. The thermodynamic parameters and errors were then calculated and averaged.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) experiments were performed with a VP-DSC and cap-DSC calorimeter, both from MicroCal™, Inc. (Northampton, MA). The protein concentration was set to 4 mg/ml. The scan rate was 1 K/min. Only the first heating scan is shown in the graphs of this study. Calorimetric traces were recorded from 10 to 95°C. However, just the temperature intervals where effects are observed are presented 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.

Results and discussion

Polysorbates are amphiphilic molecules and thus are surface active. The difference between polysorbate 20 and 80 is due to the different fatty acid (Fig. 1). Polysorbate 20 is mainly a monolaurate ester, whereas polysorbate 80 is a monooleate ester. This variation is the reason for a difference in the HLB index (hydrophilic–lipophilic balance according to Griffin 1949, 1954) with HLB(polysorbate 20) = 16.7 and HLB(polysorbate 80) = 15. Thus, polysorbate 20 is more hydrophilic compared to polysorbate 80 (Salager et al. 2002). The critical micellar range at 25°C for polysorbate 20 is approx. 50 µM and below 10 µM for polysorbate 80 (unpublished results). The interaction studies of the detergents with the proteins were performed in an identical buffer composed of 25 mM sodium citrate, 115 mM NaCl at pH 6.

Interaction of polysorbate 20 and 80 with lysozyme

The interaction of polysorbate with lysozyme was first investigated at low temperature (15°C). Exothermic (ΔH_{obs}) events are measured (Fig. 2). However, the heat effects are very small. This is illustrated exemplarily for the titration of polysorbate 20 to lysozyme. The corresponding reference measurements show also very small effects (Fig. 2). The reference enthalpy (ΔH_{ref}) contains contributions from the dilution enthalpy and demicellisation enthalpy. The reaction enthalpy (ΔH) at a defined temperature is calculated according to: $\Delta H = \Delta H_{obs} - \Delta H_{ref}$. In order to investigate whether the binding is entropically driven, and not by enthalpy, the titration experiments were performed at different temperatures between 5 and 55°C (Fig. 3), thus at temperatures below the onset of protein denaturation (Fig. 4). As can be seen from Fig. 2, with

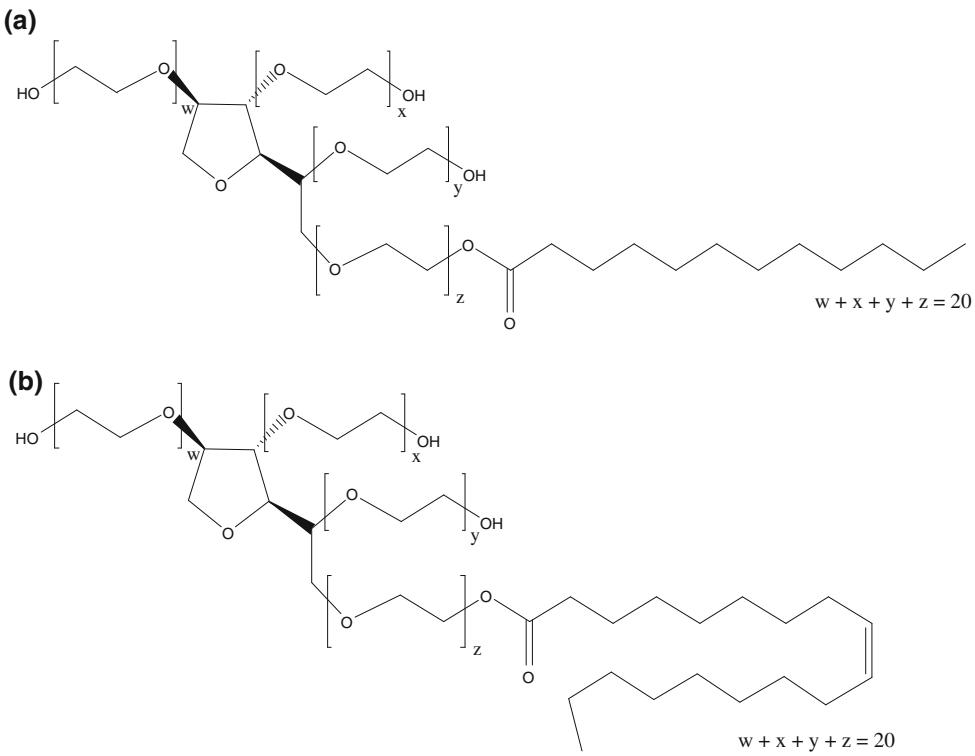

Fig. 1 Chemical structure of (**a**) polysorbate 20 (Tween® 20) and (**b**) polysorbate 80 (Tween® 80)

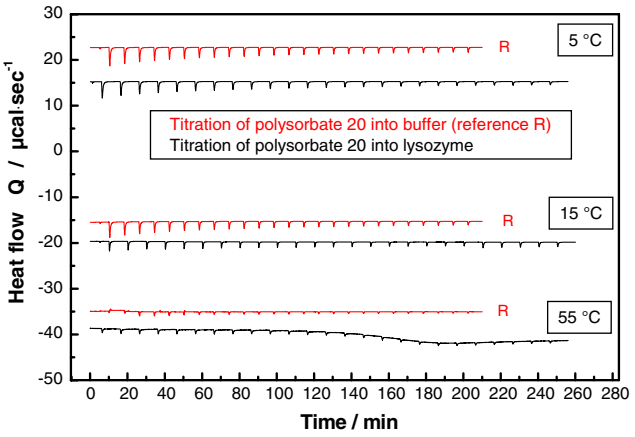

Fig. 2 Experimental power flow signals of the interaction of polysorbate 20 (10 mM) with lysozyme (1.1 mM) at three different temperatures (5, 15, 55°C). Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl. The corresponding reference experiments, titration of polysorbate 20 into buffer, are also shown (denoted by *R*)

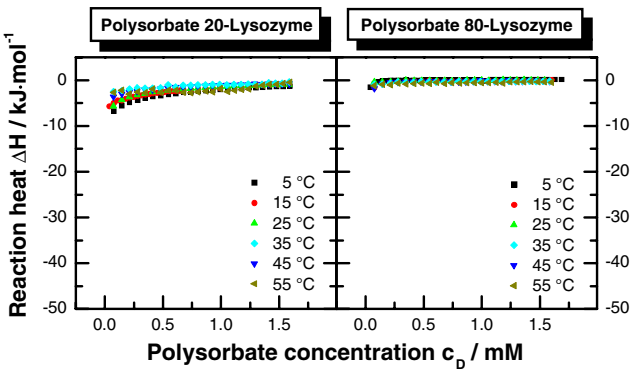

Fig. 3 Binding isotherms of polysorbate 20 and polysorbate 80 to lysozyme between 5 and 55°C. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

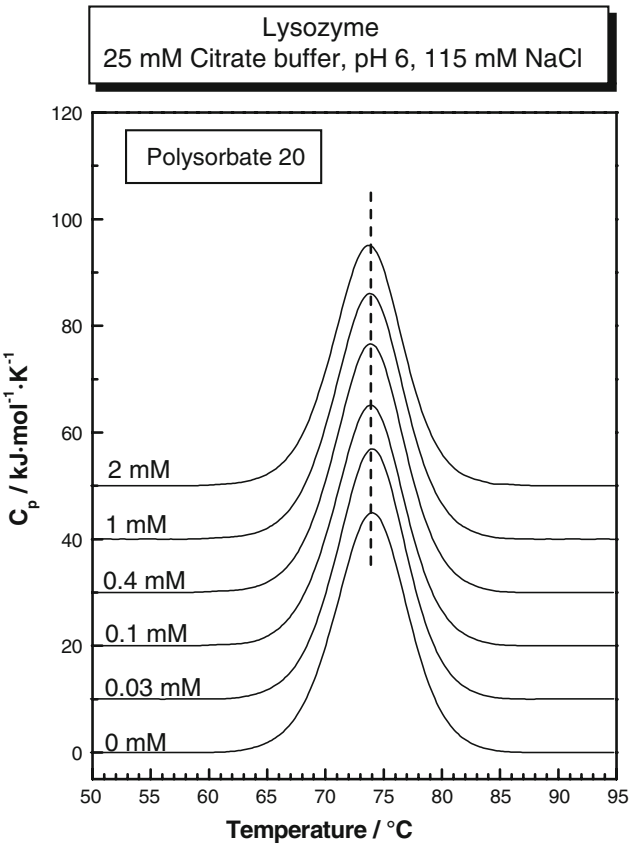

Fig. 4 Heat capacity curves (C_p) of lysozyme in the presence of different concentration (0, 0.03, 0.1, 0.4, 1, 2 mM) of polysorbate 20. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

increasing temperature the heat flow decreases. The magnitude of the reaction and dilution enthalpy is quite similar. The reaction heat for the titration of polysorbate 20 and 80 to lysozyme at different temperature are illustrated in Fig. 3. From the effects observed, it is unlikely that the detergents interact with lysozyme. This may be due to self-association of the protein under the used solution conditions of pH 6 (Sophioanopoulos and van Holde 1964; Wilson et al. 1996).

In order to see whether polysorbate 20 and 80 have an effect on the thermal stability of the protein, DSC measurements were performed for detergent concentrations ranging between 0 and 2 mM. In the absence of detergent the heat capacity curve of lysozyme is characterised by one transition with the maximum of the heat capacity curve located at 74.0°C. The denaturation enthalpy is approx. 360 kJ/mol. This is in accordance to published data (Shih and Kirsch 1995; Petersen et al. 2004; Singh and Singh 2003). The presence of polysorbate induces no change in the shape of the heat capacity curve and no change in the denaturation temperature and no change in the denaturation enthalpy. This is exemplarily shown for polysorbate 20 (Fig. 4). Thus, the presence of polysorbate 20 and 80 up to a concentration of 2 mM has no impact on the thermal stability of lysozyme. Higher detergent concentrations were not evaluated, because higher detergent concentrations can not be accepted for parentereral products.

Interaction of polysorbate 20 and 80 with bovine serum albumin (BSA)

The situation is different investigating the interaction of these detergents with BSA. A titration experiment at 25°C is shown (Fig. 5) for the system polysorbate 20-BSA. The corresponding reference experiment is included in Fig. 5. Again, exothermic events are observed, however the effects are much larger. For the first injection a heat effect of more than −30 kJ/mol is measured, compared to the very low values of −5 kJ/mol detected for the polysorbate 20-Lys system (25°C). Similar reaction enthalpies as for the system polysorbate 20-BSA have also been reported for the system

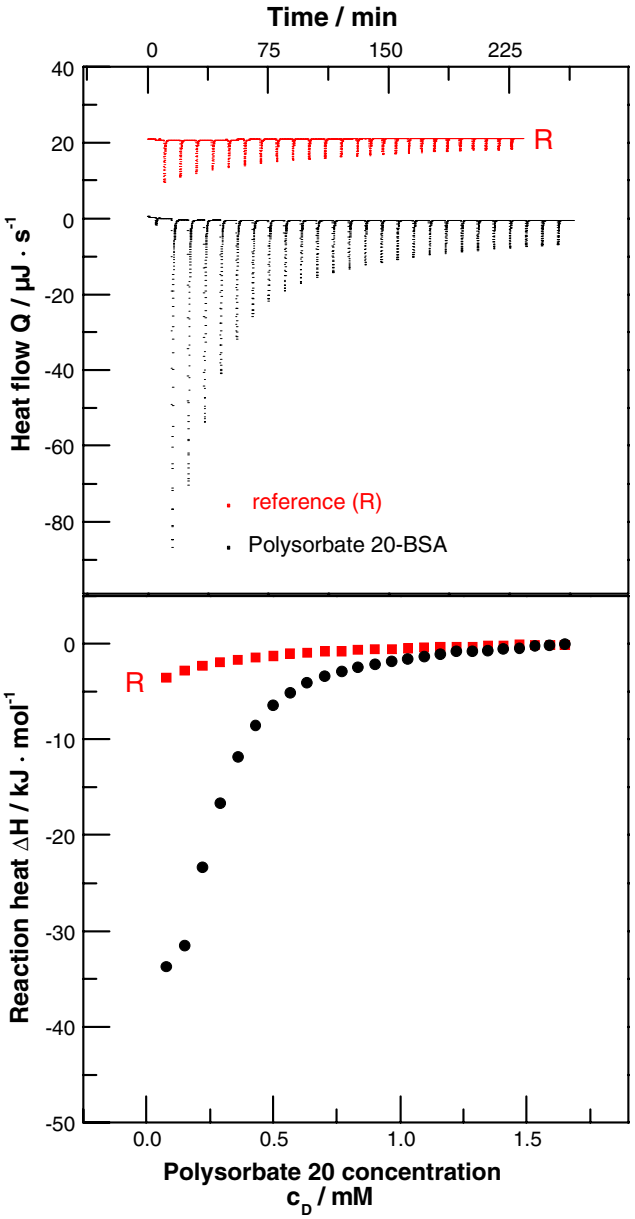

Fig. 5 Experimental power flow signals (*top*) and binding isotherm (*bottom*) (obtained by integration of the titration peaks) of the interaction of polysorbate 20 (10 mM) with bovine serum albumin (BSA) (0.24 mM) at 25°C. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl. The corresponding reference experiment, titration of polysorbate 20 into buffer, is also shown (denoted by *R*)

polysorbate 20-recombinant human growth hormone or interferon-gamma (Bam et al. 1998).

The first eight injections lead to strong exothermic effects, which become smaller the more detergent is added to the cell (Fig. 5). Beyond the 15th injection the peaks are similar to reference peaks. The shape of the titration curve for the polysorbate-BSA system is indicative for a binding reaction. Similar thermograms were obtained for the system polysorbate 80-BSA (data not shown).

Using the binding model with one set of sites (Arouri et al. 2007), the binding constant K_a at 25°C for polysorbate 20-BSA interaction is $1.2 \times 10^4 \pm 800\ M^{-1}$ and for polysorbate 80-BSA to $8.8 \times 10^3 \pm 1{,}500\ M^{-1}$. One to two detergent molecules bind per BSA molecule at 25°C. Although polysorbate 80 is to some extent more hydrophobic according to the HLB value, the affinity to BSA is slightly smaller compared to polysorbate 20. This may be due to a larger steric hindrance for the bulkier oleate fatty acid chain (polysorbate 80) upon binding to the protein. With increasing temperature the binding constant decreases. However, reaching the onset of the unfolding transition temperature the binding constant as well as the binding enthalpy increases again due to binding to already unfolded protein sequences with hydrophobic surfaces (see Fig. 7).

Chou et al. (2005) showed that the interaction of polysorbate 20 to human serum albumin (HSA) is stronger compared to the interaction of polysorbate 80 to HSA. This is in accordance to our results with BSA.

The corresponding thermodynamic data at 25°C for the system polysorbate 20-BSA are: $\Delta H^\circ = -57 \pm 2\ kJ\ mol^{-1}$, $T \times \Delta S^\circ = -30 \pm 5\ kJ\ mol^{-1}$, and $\Delta G^\circ = -23 \pm 2\ kJ\ mol^{-1}$. The thermodynamic data for the system polysorbate 80-BSA are: $\Delta H^\circ = -59 \pm 6\ kJ\ mol^{-1}$, $T \times \Delta S^\circ = -37 \pm 5\ kJ\ mol^{-1}$, and $\Delta G^\circ = -23 \pm 0.4\ kJ\ mol^{-1}$. These data indicate that the binding mechanism for the two different detergents to BSA is quite similar. The Gibbs enthalpy is negative and similar in magnitude for both interactions. A strong favourable and dominant negative binding enthalpy is obtained for both reactions. Such a binding pattern is observed for reactions involving strong van der Waals interactions and/or the formation of hydrogen bonds between the protein and the ligand (O'Brien and Haq 2004). The latter interaction is likely between the hydroxyl and/or polyoxyethylen groups of the polysorbate and the amino and carboxy groups of the protein.

However, the favourable enthalpy effect is reduced by an unfavourable entropy term leading to a smaller negative Gibbs energy of binding of $-23\ kJ\ mol^{-1}$.

The reaction enthalpy as a function of temperature is shown in Fig. 6. With increasing temperature the reaction enthalpy becomes less negative. This leads to the conclusion that the binding of detergents to BSA is temperature dependent and that entropic effects reduce the interaction of polysorbate with BSA. Comparing the binding constant at low (5°C) and high temperature (55°C) a decrease of one magnitude of order is observed, independent of the polysorbate investigated (data not shown). The thermodynamic parameters of the binding reactions, as a function of temperature, are represented in Fig. 7. The Gibbs energy change is almost temperature independent with values between -23 and $-25\ kJ\ mol^{-1}$ and the reaction enthalpy

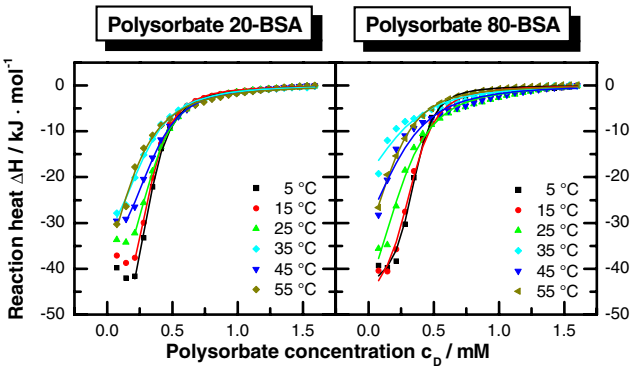

Fig. 6 Binding isotherms of polysorbate 20 and polysorbate 80 to bovine serum albumin (BSA) between 5 and 55°C. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

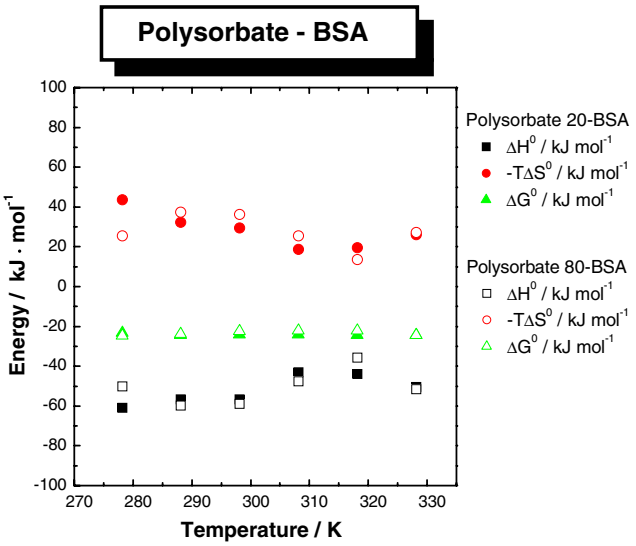

Fig. 7 Temperature dependence of the thermodynamic parameter $\Delta H°$ (change in enthalpy), $\Delta S°$ (change in entropy), and $\Delta G°$ (change in free enthalpy) for the interaction of polysorbate 20 and polysorbate 80 to bovine serum albumin (BSA). Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

decreases with increasing temperature leading to a decrease in calculated entropy change. This enthalpy signature indicates that hydrophobic interactions are not dominant (Fig. 7).

Liu et al. (2006) have used fluorescence spectroscopy for the investigation of BSA-polysorbate 20 interaction. The presence of the detergent quenches the intrinsic fluorescence of BSA (Weichel et al. 2008). With increasing polysorbate 20 concentrations, the fluorescence intensity of tryptophan decreases connected with a shift of the emission maximum wavelength from 342 nm to lower wavelengths of 334 nm (blue shift). This effect is explained by the fact that the tryptophan environment becomes more hydrophobic in the presence of polysorbate 20 due to an interaction of BSA with the detergent. Such a shift in the emission spectrum may also be related to a conformational change of the protein. Due to the fact that BSA is a fatty acid binding protein, it is expected that the acyl chain of the detergent enters the hydrophobic binding cavity of BSA and interacts with the present tryptophan residues (Liu et al. 2006). Nishikido et al. (1982) describe that Triton X-100 (non-ionic detergent) binds to BSA with a maximum of four detergent molecules per protein in a Langmuir-type binding. No gross structural changes were observed for BSA upon Triton X-100 binding. Lund et al. (2006) have shown that the anionic detergent sodium dodecyl sulphate also binds to BSA, and depending on the protein, influences denaturation kinetics (Nielsen et al. 2007a, b).

The presence of detergent has also an impact on the thermal stability of BSA (Fig. 8). In the absence of detergent, the thermogram of BSA is characterised by an endothermic peak with the maximum of the heat capacity curve located at 68.7°C and a denaturation enthalpy of ca. 870 kJ mol^{-1}. This is in accordance to reported data for similar BSA formulations (Yamasaki et al. 1990, 1991; Deep and Ahluwalia 2001). The presence of polysorbate induces a shift of the denaturation temperature (defined as the maximum of the heat capacity curve) of 1.4 K to higher temperature and an increase in the denaturation enthalpy of approx. 410 kJ mol^{-1}. Thus at a detergent concentration of 2 mM the denaturation enthalpy is ca. 1,300 kJ mol^{-1}. The DSC results show that the presence of polysorbate increase slightly the stability with regard to thermal denaturation (Fig. 8). The above mentioned effects also show that there is a direct interaction between the detergent and BSA, because there are changes in the thermodynamic parameters. This is consistent with the ITC results.

Interaction of polysorbate 20 and 80 with an immunoglobulin

Comparing the binding reaction of polysorbate to an immunoglobulin with the binding to BSA, it is obvious that the polysorbate affinity is lower for the binding to the immunoglobulin (Fig. 9). With increasing temperature the binding enthalpy decreases as observed for the polysorbate-BSA system. The polysorbate-immunoglobulin binding constant K_a is very low, i.e. in the range between 1,000 and 100 M^{-1}. Analysis of the effect of polysorbate on the thermally induced denaturation of the immunoglobulin shows that the heat capacity curves are basically not affected by the presence of polysorbates (Fig. 10). The thermogram of the immunoglobulin is characterised by two endotherms resulting from the difference in thermal stability of the Fab and Fc parts of the immunoglobulin (Ionescu et al. 2008). A very small increase of the maximum of the heat capacity curve of 0.2 K is observed in the presence of polysorbates. The overall transition enthalpy of the pure immunoglobulin

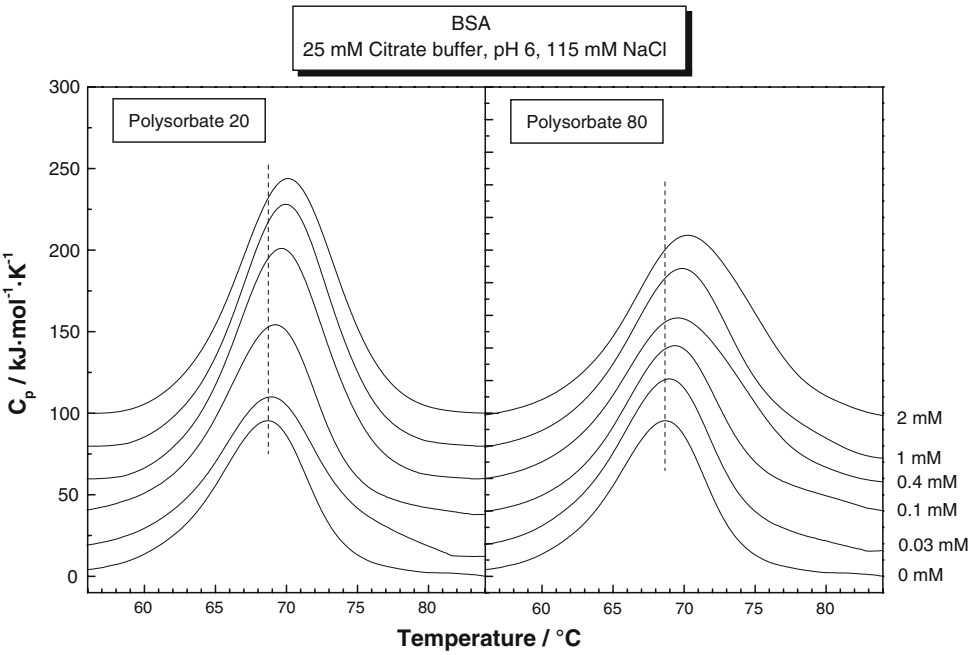

Fig. 8 Heat capacity curves (C_p) of bovine serum albumin (BSA) in the presence of different concentration (0, 0.03, 0.1, 0.4, 1, 2 mM) of polysorbate 20 (*left*) and polysorbate 80 (*right*). Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

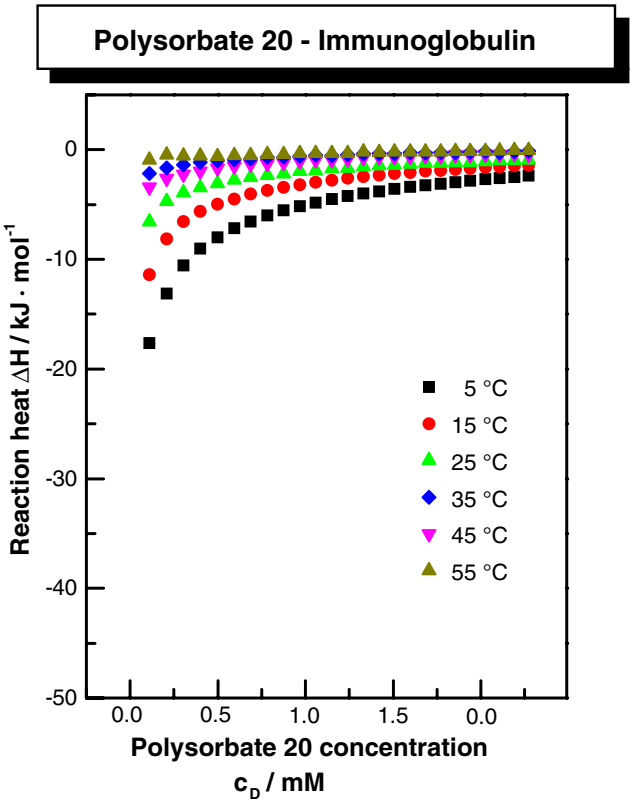

Fig. 9 Binding isotherms of polysorbate 20 to an immunoglobulin between 5 and 55°C. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

is approx. 2,700 kJ mol^{-1} for the analysed formulation conditions and also quite unaffected by the presence of polysorbate. Similar results were obtained for polysorbate 80 (data not shown). These results lead to the conclusion that the interaction of polysorbates with the investigated immunoglobulin is negligible.

Protein–polysorbate systems and impact on protein stability

In a stirring study the concentration effect of polysorbate 20 with regard to the ability to stabilise the immunoglobulin against aggregation was analysed. The aggregation of the protein was followed by measuring the turbidity of the protein solution (Schüle et al. 2007; Mahler et al. 2005). Stirring the protein sample at 600 rpm at 25°C (according to the procedure described in Mahler et al. 2005) in the absence of polysorbate induces after 48 h of stirring a strong increase of the turbidity from 3 FNU to 43 FNU (Formazin Nephelometry Units) indicating an extensive formation of aggregates. The presence of polysorbate 20 at concentrations of 0.1 and 0.4 mM reduces strongly the formation of aggregates; the turbidity is now in the range of 5–6 FNU. However, at a polysorbate 20 concentration of 2 mM an increase in turbidity up to 25 FNU is again observed. It is described, that protein aggregation may also be favoured sterically by detergent binding by way of the flexible hydrocarbon chain (Nishikido et al. 1982). This shows that polysorbate 20 is able to protect the immunoglobulin against the formation of aggregates induced by stirring. However, the effect is detergent concentration dependent.

Bam et al. (1998) showed that the presence of polysorbate protects recombinant human growth hormone against agitation induced damage via hydrophobic interactions. This is especially observed, for polysorbate-protein molar ratios >4.

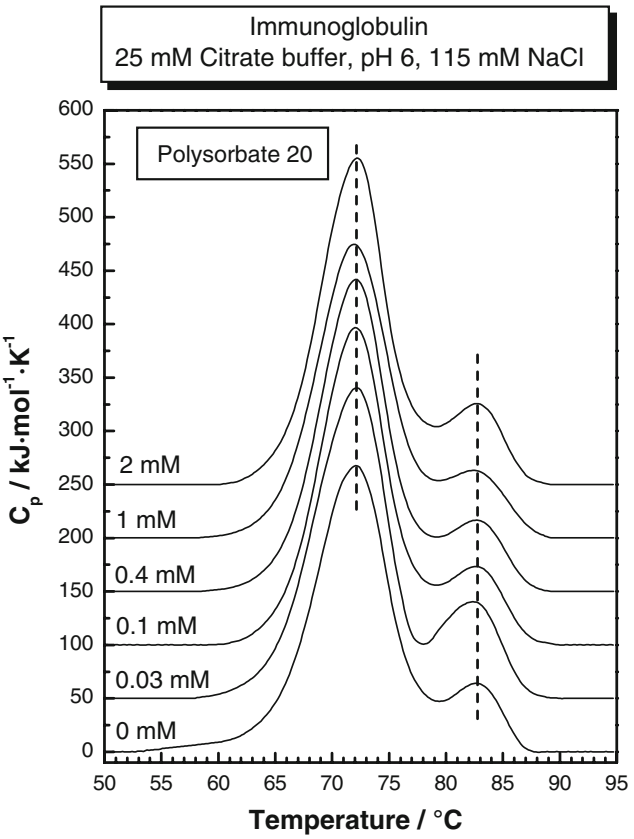

Fig. 10 Heat capacity curves (C_p) of an immunoglobulin in the presence of different concentration (0, 0.03, 0.1, 0.4, 1, 2 mM) of polysorbate 20. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

On the other hand, Wang et al. (2008) have shown, that polysorbate 80 has a dual stabilisation effect: stabilisation is observed against the formation of aggregates via physical stress, but promotes the formation of aggregates in accelerated stability studies.

Conclusions

Polysorbates are used to stabilise proteins against the formation of aggregates. However, the stabilisation mechanism is quite unclear and the effect of polysorbate with regard to its stabilisation property can not be predicted.

Although, polysorbate 20 is able to stabilise the immunoglobulin against the agitation induced aggregate formation, there seems to be no direct interaction between the detergent and the immunoglobulin, i.e. there is no specific protein to detergent ratio requested for the stabilisation effect. The binding affinities as determined by ITC are quite low ($\ll$1,000 M^{-1}). The extremely weak binding is confirmed using intrinsic tryptophan fluorescence spectroscopy (data not shown). Furthermore, the effect of polysorbate on the thermal stability is marginal, as shown by the small changes of the DSC heat capacity curves of the immunoglobulin-detergent systems compared to the pure immunoglobulin solutions. Similar results were observed for the system polysorbate-lysozyme, where the interaction is also negligible. Thus, a possible stabilisation effect of polysorbates could be explained by the so-called "preferential exclusion and hydration" mechanism (Bhat and Timasheff 1992; Timasheff 1998; Shimizu and Smith 2004). However, this has to be confirmed by appropriate investigations.

For BSA the situation is different. This is summarised in Fig. 11. Binding is observed for the two polysorbates to BSA. The reaction is enthalpically driven. One to two polysorbate molecules bind to BSA. The presence of polysorbate has also a positive effect with regard to the stabilisation of the protein structure with regards to thermal induced denaturation.

However, compared to the binding reaction of e.g. immunoglobulins to protein A, where binding constants in the range of 10^7–10^8 M^{-1} are observed (Arouri et al. 2007), the binding constants of polysorbate to BSA are quite low

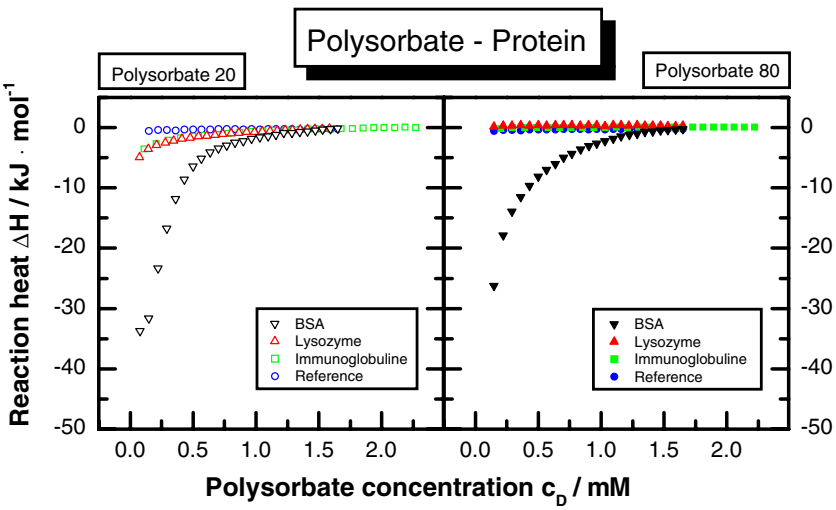

Fig. 11 Comparison of the binding isotherms of polysorbate 20 and polysorbate 80 to various proteins: bovine serum albumin (BSA), lysozyme and immunoglobulin at 25°C. The reference experiment is also shown. Buffer composition: 25 mM sodium citrate, pH 6, 115 mM NaCl

with about 4–5 orders of magnitude lower binding constants.

The aim of the study was to investigate whether there is a direct binding of polysorbate to proteins. This was assessed by isothermal titration and differential scanning calorimetry. The advantage of ITC is that a complete set of thermodynamic parameters of the interaction can be directly measured in a variety of ways, which provide insight into the stability, specificity and stoichiometry of several biomolecular interactions. The results show for the three investigated proteins, lysozyme, bovine serum albumin and an immunoglobulin, that polysorbate 20 and 80 binding is different depending on the analysed protein. No significant binding to lysozyme and the investigated immunoglobulin is observed, whereas binding is detected for bovine serum albumin. The beneficial effect of polysorbate for the avoidance of the formation of aggregates for immunoglobulin therapeutics cannot directly be linked to a strong polysorbate-immunoglobulin binding reaction. Other effects seem to be more relevant. These effects have to be investigated in future studies, in order to understand the "polysorbate anti-aggregation effect" on immunoglobulins.

Acknowledgments We thank Heidrun Schott for continuous technical support, and Stefan Bassarab for his ongoing interest and support.

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