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Investigation of adsorption-induced structural changes of proteins at solid/liquid interfaces by differential scanning calorimetry

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Abstract

Adsorption of proteins on solid surfaces is widely studied because of its importance in various biotechnological, medical, and technical applications, e.g. biosensors, cardiovascular implants and chromatography.

One of the main problems is to what extend adsorption-induced conformational changes occur since they often modify the biological activity of the protein. Thus, the investigation of structural rearrangement due to interaction with the solid surface is of great interest. As protein molecules are in many cases continuously exchanged between the adsorbed and dissolved states, the question arises whether protein molecules re-adopt their original native structure after release from the surface.

In this study, two model proteins with well-characterized properties (human serum albumin (HSA) and α-chymotrypsin) were adsorbed from aqueous buffered solution onto finely dispersed hydrophilic silica particles. Adsorption isotherms were determined from the depletion of the supernatant, protein concentrations were analyzed by photometric methods. Reversibility of the processes was tested.

The structural rearrangements in the protein molecules induced from the adsorption process were probed by highly sensitive differential scanning calorimetry (micro-DSC). Transition temperature and enthalpy measurements for thermal unfolding were compared between given amounts of protein in free (native) and adsorbed/desorbed form.

From these measurements it appears that under the chosen adsorption conditions, HSA may lose essentially all of its cooperatively folded structure, while α-chymotrypsin retains full structure. The relation between the native protein structural stability on the one hand, and structural rearrangement on the other is discussed. Finally, the importance of conformational changes for spontaneous adsorption to occur is considered.

Keywords: Protein adsorption; Solid/liquid interface; Silica; Differential scanning calorimetry; Conformational changes

1. Introduction

There is a general tendency for proteins to accumulate at solid/liquid interfaces [1]. Protein adsorption can be a desired event, such as, e.g. in biosensor applications [2] and immunoassays [3], as well as in protein-purification strategies and in drug delivery systems [4]. In other situations, however, it is an unwanted process. Negative consequences of protein adsorption include activation of coagulation and immune response upon blood contact with artificial implant materials, loss of performance of kidney dialysis membranes and oxygenators, and plaque formation on teeth [5]. An effective control of any of these processes requires a detailed understanding about the interactions.

A crucial aspect of protein/surface phenomena is the effect of surface adsorption on protein structure and consequently on biological activity (structure–

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function relationship). Just as solvent environments may impact the structure of proteins, so may surfaces with which they come in contact. Limited evidence exists that adsorbed proteins may become conformationally altered on some surfaces, although the degree and nature of the conformational changes are often not understood (e.g. [6]). This is due to both the intrinsic difficulties in studying proteins on solid/liquid interfaces and the low resolution of the methods that generally have been employed to characterize the conformation of the adsorbed protein.

Several evidences for structural changes in the adsorbed protein molecules are indirect, such as (endothermic) shifts in initial slopes of the adsorption isotherms as a function of temperature [7], layer thicknesses smaller than the shortest axis of the native molecule [8], and from reductions in biological activity upon adsorption [9].

In order to obtain direct structural information for the adsorbed protein molecules various techniques have been used. The most common are spectroscopic methods such as (total internal reflection) fluorescence [10], Fourier transform infrared spectroscopy (attenuated total reflection) [11,12], circular dicroism (CD) [13–15], and XPS [16]. Lu et al. [17] studied the denaturation of lysozyme layers adsorbed at a hydrophobic silicon/water interface using specular neutron reflection. Recently, McNay and Fernandez [18] have used nuclear magnetic resonance (NMR) spectroscopy and amide proton–deuteron exchange techniques to study the unfolding of lysozyme adsorbed on reversed-phase supports. Sane et al. characterized proteins adsorbed on standard chromatographic materials by means of amide I band Raman spectroscopy [19].

A potentially sensitive method for exploring the structure of proteins both in solution and adsorbed to surfaces is in terms of their conformational stability. For example, valid information on protein stability in solution can be obtained from the thermogram of unfolding [20–22]. The unfolding of proteins is generally an endothermic process that can be studied by DSC. From the transition temperature (T_d), the width of the transition, and the area under the heat capacity curve, one can assign a relative stability to the selected protein in a particular solvent environment. It follows that a comparison of the stability of proteins on and off a surface should provide a sensitive probe of protein–surface interaction. This was shown in previous studies [23–26]. The stability of proteins in solution is most often assessed by disrupting the native-state with denaturing agents, or by moving the pH or temperature. Steadman et al. [25] have found, however, that only temperature perturbation does not significantly alter the amount of protein on most surfaces.

In conclusion, certain aspects of protein adsorption at solid/liquid interfaces are still controversial, in particular, the reversibility of the process, and the conformation of the adsorbed protein molecule. The present study was aimed at clarifying these questions for two selected model cases.

This paper considers the adsorption characteristics of human serum albumin (HSA) and α-chymotrypsin on solid/liquid interfaces under varying adsorption conditions. Careful measurements of the adsorption and desorption isotherms were carried out. The relationship between protein solution stability and structural retention upon adsorption was examined. Data have been obtained using differential scanning calorimetry of the native protein, as well as of the protein adsorbed onto and desorbed from the surface. DSC was used as it provides a basis for an assessment of the overall structure of the protein in solution and on the solid surface, whereas many spectroscopic methods only enable to highlight local rearrangement. The solid surfaces used in this investigation were two different types of silica particles with a large effective surface area. That enables them to be used in methods which require a high protein concentration such as in the present study DSC.

The model systems examined in the present work refer in part to earlier investigations by other authors. This offers the possibility to compare the results of the experiments established in this study.

2. Experimental

2.1. Materials

2.1.1. Model proteins and their properties

HSA was purchased as the highest grade available from Sigma (approximately 99%, A-3782, Taufkirchen, Germany). The structure of HSA has been determined by X-ray crystallography of high resolu-

Table 1
Properties of the model proteins

Protein	Molecular weight (Da)	Dimensions (nm)	IEP	Secondary structure	Function
HSA[a]	66000	15 × 3.8 × 3.8	4.7	α-helix (48%), β-sheet (15%)	Blood plasma protein, regulation of osmotic pressure, protein reserve, transportation agent
α-chymotrypsin	25200	5.1 × 4 × 4[b]	8.1[c]	α-helix (11%), β-sheet (51%)[d]	Mammalian digestive enzyme, catalyses the hydrolysis of proteins in the small intestine hydrolyzes ester bonds in addition to peptide bonds

[a] [56].
[b] [57].
[c] [58].
[d] [59,60].

tion [27]. It has three homologous domains that assemble to form a heart-shaped molecule.

Crystalline α-chymotrypsin from bovine pancreas (lyophilized, 102307) was supplied by Merck (Germany). The proteins were used without further purification.

Important properties of the proteins are given in Table 1.

2.1.2. Adsorbents and their properties

Aerosil OX-50 (Degussa, Germany) is a powdered pyrogenic non-porous hydrophilic silica with a specific surface area of $48.2 \pm 0.5\ m^2/g$. The primary particles have a mean diameter of 40 nm. The Spherosil XOB 015 M (Rhône-Poulenc, France) is a hydrophilic precipitated silica with a specific surface area of $29.4 \pm 0.3\ m^2/g$. The mean diameter of the porous particles is 47 μm ($D(v, 0.5)$; Mastersizer, Malvern Instruments, UK), their size is ranging between 10 and 100 μm. Pore size distribution showed an average porosity of 300 Å [28]. The specific surface areas of the solids have been determined by volumetric adsorption of nitrogen at 77 K (BET-Liquisorb-EL, Porous Material Incorporated, USA) and by applying the BET method with the molecular cross-sectional surface area of nitrogen assumed to be 16.2 $Å^2$.

The point of zero charge of silica is pH 2–3. Thus, the silica particles have a negative charge at the pH of the experiments.

2.1.3. Other chemicals

Two types of buffer were used: PBS (phosphate buffered saline tablets: 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4; Sigma, Germany) and 0.01 M sodium/potassium phosphate buffer (Na_2HPO_4 (>99%, ACS reagent, Aldrich, Germany), KH_2PO_4 (>99%, ACS reagent, Aldrich, Germany), pH 7.1). All water was purified using a Millipore water filtering system (Milli-Q, Millipore, Germany).

2.2. Adsorption isotherms

The adsorption experiments were performed in 2 ml tubes (Eppendorf, Germany) each containing equal amounts of adsorbent material (Aerosil: 10 or 30 mg; Spherosil: 17 or 50 mg). Equal volumes (2 ml) of protein solutions varying in concentration were added to the adsorbent. In some experiments the protein concentration was adjusted by adding calculated volumes of buffer and protein stock solution to the adsorbent. These different ways of putting adsorbent and protein solution together did not cause any differences in the adsorption isotherms. The protein–particle suspension was gently mixed by careful end-over-end rotation to minimize exposure of protein to air–solvent interface, and allowed to incubate at 22 °C for 1–16 h. Preliminary experiments had shown that, in the case of adsorption, 1–2 h was sufficient time for a constant value to be reached.

The tubes containing the protein and adsorbent were then centrifuged (Centrifuge 5403, Eppendorf, Germany; 15,000 r/min; 15–30 min; 22 °C) and the residual protein concentration in the supernatant was determined spectrophotometrically (UV–VIS spectrometer Specord S10, Analytik Jena GmbH, Germany) by absorbance at 280 nm. Low protein concentrations (<0.1 mg/ml) were additionally determined by

a colorimetric method (modified Bradford method [29,30]) using the Roti®-Nanoquant protein assay (Roth, Germany) in combination with a microplate reader (Anthos Reader 2010, Anthos Labtec Instruments GmbH, Austria).

The amount of bound protein (Γ) was calculated from the difference in the protein concentration before and after adsorption (protein mass balance). It is plotted against the protein concentration in solution after adsorption c_e. Correction was made for protein adsorption to tube surfaces, pipettes, etc. by parallel incubation and handling of protein standards without the surface (in most cases the adsorbed amount was negligible). All determination were conducted at least three times.

All glassware was cleaned with sulfur chromic acid before use.

2.3. DSC measurements

The same procedure as for adsorption isotherms was used to prepare the protein–particle complexes for calorimetric analysis experiments. After incubation, the protein-covered particles were washed three times by centrifuging the protein–particle suspension, decanting the supernatant, and redispersing in protein-free buffer. The pelleted silica surface was used within 24 h for DSC studies. Results were found to be independent of time over the range of storage times (0.5–24 h) examined.

The proteins dissolved in the supernatant after the adsorption experiment had been in contact with the large adsorbent-surface area for an extended time (adsorption/desorption equilibrium). During incubation, homomolecular exchange of protein molecules between the hydrophilic surface and the solution is expected to take place [31,32], so that after a period of 16 h, most, if not all, of the proteins had been adsorbed at the surface for some time. In order to study whether these proteins may change their conformational structure compared to the native-state, too, the supernatant was also studied by DSC.

Differential scanning calorimetry was performed using a Setaram micro-DSC III heat conduction scanning microcalorimeter (Setaram, France). Protein (0.8–5.1 mg), either in the adsorbed-state or in solution (native or dissolved in supernatant after adsorption experiment), were placed into the 1 ml measurement ampoule (Hastelloy C). Absorbed or released heat was recorded relative to the 1 ml reference ampoule filled with an appropriate blank sample (adsorbent dispersion or buffer solution). The ampoules were allowed to stabilize at 25 °C for 60 min prior to the initiation of the scanning experiment over the temperature range of 25–110 °C. The heating rate was 0.5 K/min for all experiments. The surface materials themselves produced no evidence of endothermic or exothermic transitions over the temperature range examined. Cooling of the ampoule to the initial conditions and rescanning the sample indicated that the protein transitions were not reversible.

The second run of the same sample was used as the background. All measurements were repeated at least once.

The enthalpy of the transition ($\Delta_d H$) was calculated from the area under the peak. According to Privalov [33], denaturation enthalpies correspond to the loss of favorable intramolecular interactions within the protein molecule. Denaturation temperature, T_d, is defined as the temperature at which a local maximum occurs in the excess heat capacity.

The concept of using DSC to study adsorption-induced denaturation is based on the idea that if the protein has been denatured upon adsorption/desorption, thermal denaturation will not be observed. Therefore, the conformation of the adsorbed/desorbed protein is considered totally or partly denatured if the expected melting transition of the protein is not observed or reduced. It is assumed that heat denaturation of the adsorbed molecules leads to similar denatured state as in solution. T_d reflects the stability of the protein molecule. Thus, interaction of proteins with solid/liquid interfaces may also result in a shift of the denaturation peak.

2.4. Desorption

For reversibility measurements, known weights of clear supernatant were removed after centrifugation and replaced by the same weights of buffer solution, followed by mixing and centrifugation as before. Then, the supernatant was examined by UV for the presence of desorbed proteins. A contact time of 5 and 16 h, respectively, was used. The desorption curves are not meant to show equilibrium values.

Also in order to make sure that all effects recorded in the DSC measurements were entirely due to surface-bound proteins it was important to check for the occurrence of desorption of pre-adsorbed protein upon dilution in buffer.

3. Results

3.1. Adsorption isotherms

The two proteins examined were rapidly adsorbed onto the silica surface. Fig. 1 shows the adsorption isotherm for HSA on Spherosil XOB 015 M in PBS after 16 h incubation time. The adsorption isotherm obtained after 1 h incubation time looks equal.

For α-chymotrypsin the different adsorption conditions result in different adsorption isotherms. Ionic strength of the buffer, adsorbent/solution ratio, and silica materials have been varied. At high ionic strength of the buffer (PBS) the adsorbent/solution ratio has no significant effect on the adsorption isotherms (Fig. 2a and b, circles). At low ionic strength (10 mM phosphate buffer), however, the adsorbed protein amount reaches higher values for lower adsorbent/solution ratio (Fig. 2a and b, triangles). If one compares the isotherms for adsorption on Spherosil XOB 015 M and Aerosil OX-50 obtained under the same experimental conditions for approximately the same accessible adsorbent-surface area, the kind of silica does not seem to have a large influence on the results (equal symbols in Fig. 2a and b).

3.2. Desorption

In all systems, desorption upon dilution was below the detectable limit, independent of adsorption and desorption times (Fig. 1, e.g. for HSA). This observation held true, regardless of the level of protein saturation of the surface.

3.3. Differential scanning calorimetry

Adsorption/desorption measurements indicated that, once bound, the protein was not released from the surface during the time course of all experiments. Therefore, it appears that the transitions described below were due entirely to surface-bound protein.

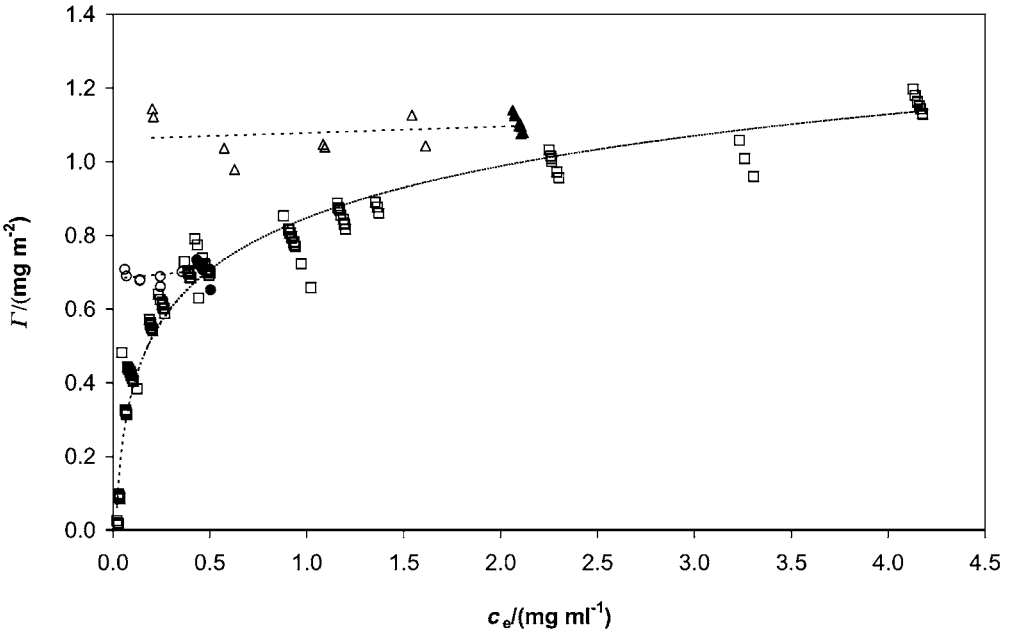
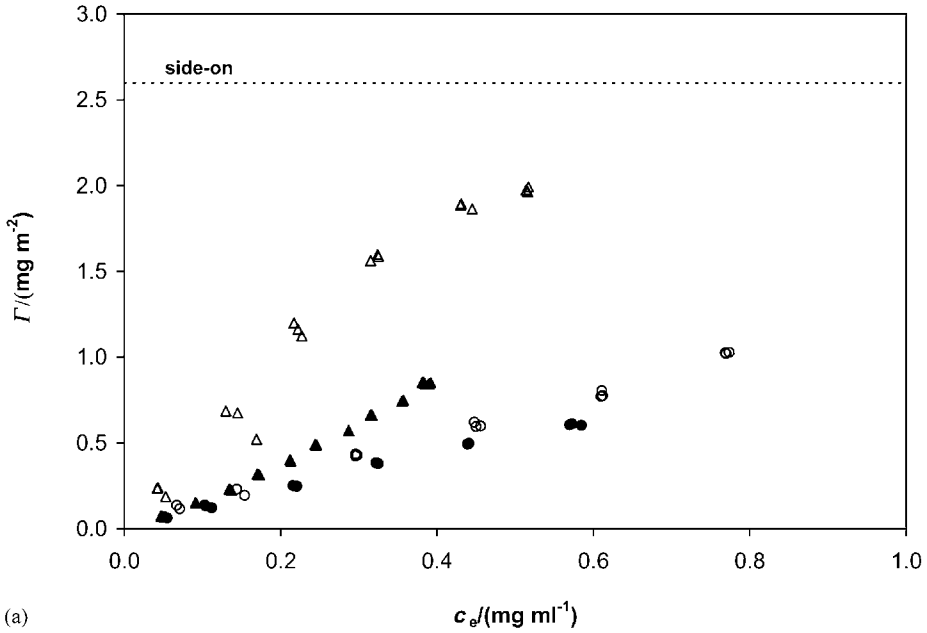
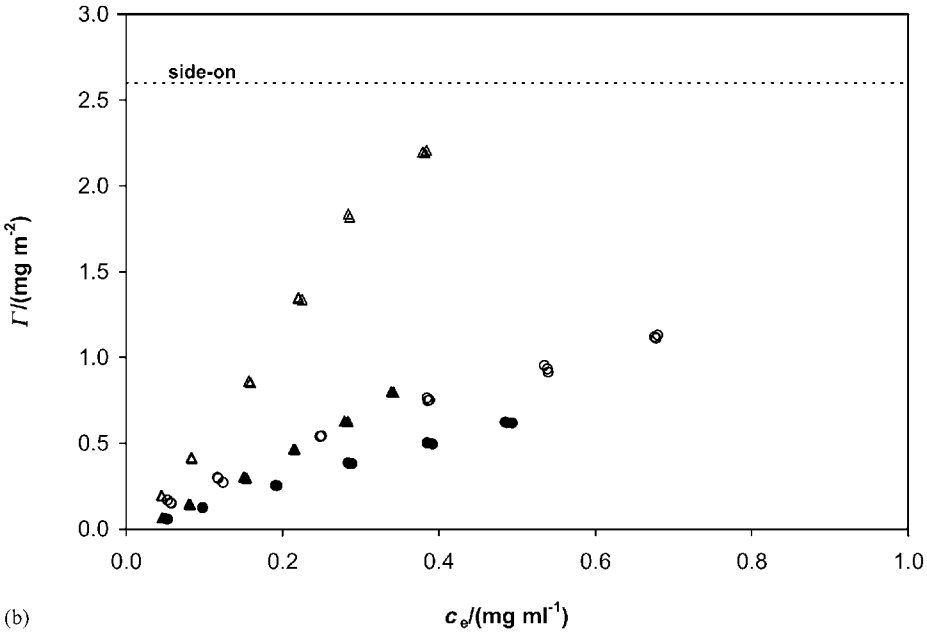


Fig. 1. Adsorption isotherm of HSA on Spherosil XOB 015 M: $T = 22\,^{\circ}C$; PBS (pH 7.4); incubation time, 16 h; 25 mg silica/ml dispersion (squares). Desorption isotherms for two different initial surface concentrations (triangles, circles); contact time, 5 h.



(a)



(b)

Fig. 2. (a) Adsorption isotherms of α-chymotrypsin on Aerosil OX-50 ($T = 22\,^{\circ}C$; incubation time, 16 h) obtained for various experimental conditions: 10 mM phosphate buffer pH 7.1 (triangles); PBS pH 7.4 (circles); adsorbent/solution ratio 15 mg/ml (filled symbols); adsorbent/solution ratio 5 mg/ml (open symbols). The horizontal dotted line illustrates the surface concentration value calculated for a closely packed monolayer of side-on adsorbed α-chymotrypsin molecules. (b) Adsorption isotherms of α-chymotrypsin on Spherosil XOB 015 M ($T = 22\,^{\circ}C$; incubation time, 16 h) obtained for various experimental conditions: 10 mM phosphate buffer pH 7.1 (triangles); PBS pH 7.4 (circles); adsorbent/solution ratio 25 mg/ml (filled symbols); adsorbent/solution ratio 8.5 mg/ml (open symbols). The horizontal dotted line illustrates the surface concentration value calculated for a closely packed monolayer of side-on adsorbed α-chymotrypsin molecules.

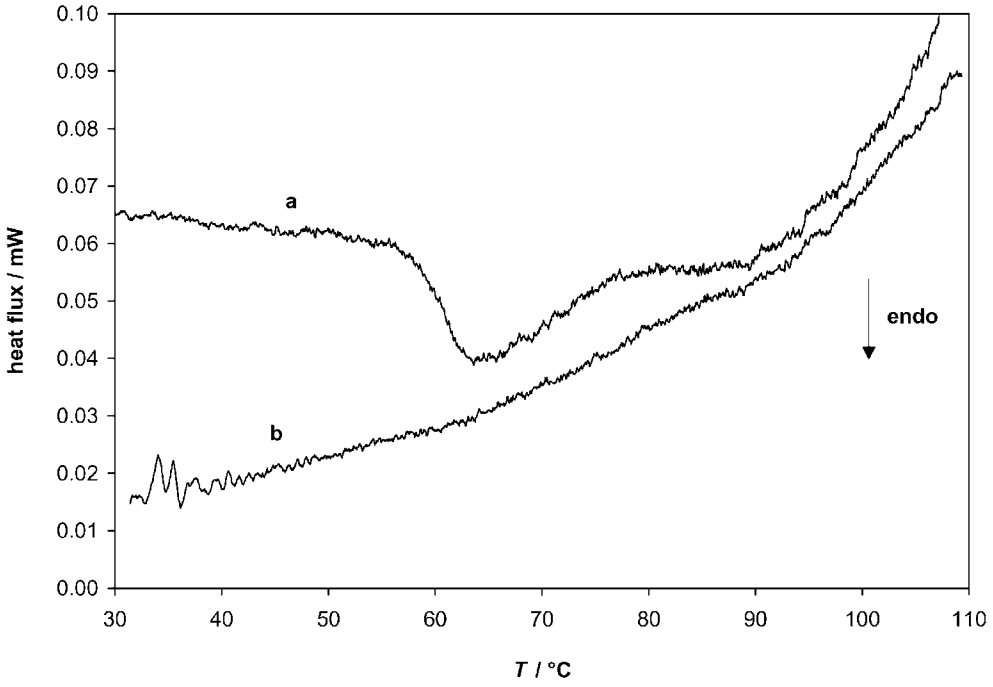


Fig. 3. Thermal stability of HSA: (a) in free form (PBS, $c = 3$ mg/ml, $m = 2$ mg); (b) in adsorbed form (Spherosil XOB 015 M, PBS, pH 7.4, $\Gamma = 1.1$ mg/m^2, $m = 3.3$ mg).

Fig. 3 illustrates typical thermograms of comparable amounts of free and adsorbed HSA in PBS. Native HSA in solution ($c = 2$–5 mg/ml) shows a single endotherm; denaturation occurs at 65 °C with a specific transition enthalpy of 12 J/g. At higher concentration the endothermic peak is followed by an exothermic one, which obviously results from aggregation. This is confirmed by visual inspection of the albumin in the calorimeter ampoule after the experiment which has become a turbid medium. The T_d value obtained in the present report is in agreement with those reported by other authors (100 mM phosphate buffer, pH 7.4: 63.1 ± 0.4 °C [34]; 150 mM NaCl, pH 7: 65–66 °C [35]). HSA exists in a compact form between pH 4.3 and 10 with a high cooperativity between the various domains [34]. The enthalpy value lies between the values (5.6 J/g [34] or 16 J/g [35]) for pH 7. A direct comparison is difficult as the denaturation parameters sensitively depend upon the solvent environment of the protein.

From the striking absence of heat capacity features in the DSC thermogram of HSA adsorbed to Spherosil XOB 015 M ($\Gamma = 1.1$ mg/m^2) it is clear that all structure in the native protein which could be collapsed though the cooperative melting process had already been eliminated from the adsorbed form as a result of its interaction with the substrate.

Since, as a rule, retention of biological activity will increase with increased protein close-packing [36], it is entirely possible that adsorption conditions may be found which allows HSA to retain some amount of structure. Also contact time between protein and substrate may have an influence on the structural perturbations. The DSC data for HSA are listed in Tables 2 and 3. Specific denaturation enthalpy, and denaturation temperatures are given for the protein in solution as well as in adsorbed-state for various adsorption conditions. It can been seen that irrespective of the surface concentration no transition peak was detected for the adsorbed HSA. Even after rather short adsorption times (1 h) HSA does not show a thermal denaturation anymore.

In Fig. 4, the DSC curves for comparable amounts of the protein α-chymotrypsin in free and adsorbed (Aerosil OX-50) form are presented. Here, the presence of the substrate appears to have little effect on

Table 2
DSC results for the system HSA–Spherosil XOB 015 M[a]

	$\Delta_d H$ (J/g)	T_d (°C)	$m_{protein}$ (mg)
Native (in solution), c = 2–5 mg/ml	12 ± 1	65 ± 1	1.6–4.1
Adsorbed (25 mg silica/ml)			
Spherosil XOB 015 M, PBS, 16 h, Γ = 1.2 mg/m^2	–	–	5.1
Spherosil XOB 015 M, PBS, 16 h, Γ = 1.1 mg/m^2	–	–	3.3
Spherosil XOB 015 M, PBS, 16 h, Γ = 0.85 mg/m^2	–	–	3.7
Spherosil XOB 015 M, PBS, 16 h, Γ = 0.7 mg/m^2	–	–	2.1
Spherosil XOB 015 M, PBS, 16 h, Γ = 0.4 mg/m^2	–	–	1.9

[a] Medium: PBS (pH 7.4); variation of the adsorbed amount.

Table 3
DSC results for the system HSA–Spherosil XOB 015 M[a]

	$\Delta_d H$ (J/g)	T_d (°C)	$m_{protein}$ (mg)
Native (in solution), c = 2–5 mg/ml	12 ± 1	65 ± 1	1.6–4.1
Adsorbed (25 mg silica/ml)			
Spherosil XOB 015 M, PBS, 16 h, Γ = 1.1 mg/m^2	–	–	3.3
Spherosil XOB 015 M, PBS, 1 h, Γ = 1.1 mg/m^2	–	–	3.3

[a] Medium: PBS (pH 7.4); variation of the incubation time.

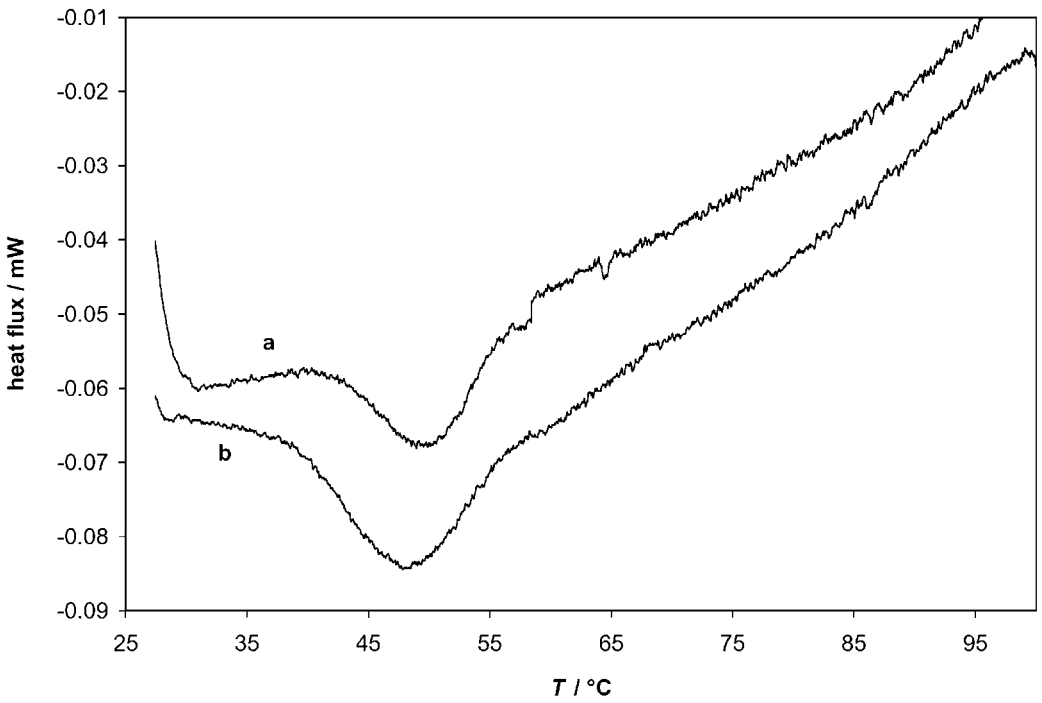


Fig. 4. Thermal stability of α-chymotrypsin: (a) in free form (PBS, c = 1 mg/ml, m = 0.8 mg); (b) in adsorbed form (Aerosil OX-50, PBS, pH 7.4, Γ = 0.5 mg/m^2, m = 1.4 mg).

Table 4
DSC results for the various systems of the α-chymotrypsin–silica[a]

	$\Delta_d H$ (J/g)	T_d (°C)	$m_{protein}$ (mg)
Native (in solution), $c = 1$ mg/ml	20 ± 1	50 ± 1	0.8
Adsorbed			
Aerosil OX-50, PBS	21 ± 1	48 ± 1	1.4
Aerosil OX-50, 10 mM phosphate buffer	20 ± 1	50 ± 1	1.4
Spherosil XOB 015 M, PBS	(12)	47 ± 1	1.7
Spherosil XOB 015 M, 10 mM phosphate buffer	(11)	51 ± 1	1.5

[a] $\Gamma = 0.5$ mg/m^2; incubation time: 16 h.

the protein structure, to judge from T_d of the major transition as well as from the enthalpy of unfolding which both remain essentially unchanged by the adsorption. Although the adsorption isotherms obtained for different substrates and buffers of different ionic strength vary substantially, these different adsorption conditions do not seem to influence the transition temperature of the adsorbed α-chymotrypsin (Table 4 for $\Gamma = 0.5$ mg/m^2). The determination of the transition enthalpies for α-chymotrypsin adsorbed to Spherosil XOB 015 M was difficult and inaccurate

Table 5
DSC results for the system α-chymotrypsin–Aerosil OX-50[a]

	$\Delta_d H$ (J/g)	T_d (°C)	$m_{protein}$ (mg)
Native (in solution), $c =$ 1 mg/ml	20 ± 1	50 ± 1	0.8
Adsorbed			
$\Gamma = 0.2$ mg/m^2	21 ± 1	50 ± 1	1.0
$\Gamma = 0.5$ mg/m^2	20 ± 1	50 ± 1	1.4
$\Gamma = 1.2$ mg/m^2	19 ± 1	49 ± 1	1.7

[a] Medium: 10 mM phosphate buffer pH 7.1; incubation time: 16 h; variation of the adsorbed amount.

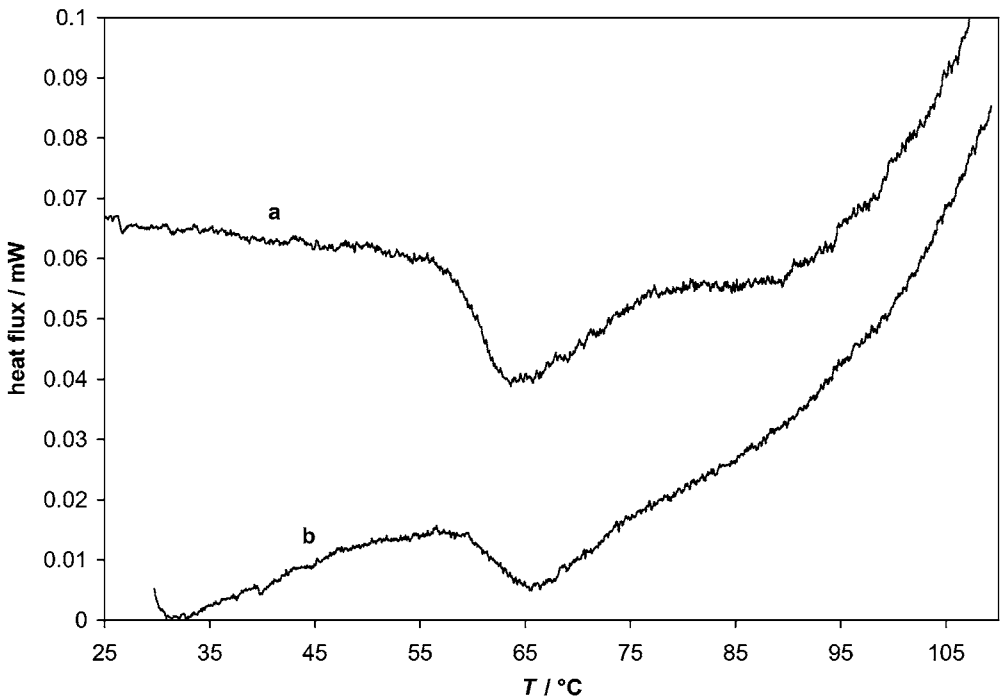


Fig. 5. Thermal stability of HSA: (a) in free form (PBS, $c = 3$ mg/ml, $m = 2$ mg); (b) in solution after being released from the Spherosil XOB 015 M surface by homomolecular exchange (PBS, $c = 1.8$ mg/ml, $m = 1.4$ mg).

due to the bad signal to noise ratio. Surface concentration of the adsorbed protein does not seem to affect its structure, as may been seen in Table 5, for example, for the adsorption onto Aerosil OX-50 in 10 mM phosphate buffer. The data for thermal unfolding of native α-chymotrypsin in solution agree well with those given by Zoungrana et al. [24] (10 mM phosphate buffer, pH 7: 49 °C; 20.4 J/g).

For each protein, the observed transitions were irreversible by the criteria that return of the sample to 25 °C and rescanning produced no detectable endotherm. The way of interpreting such irreversible process on the basis of equilibrium thermodynamics is a controversial subject. However, many authors agree on the fact that irreversible alteration of the unfolded molecules, e.g. aggregation, separation of chains, etc. usually have a much lower enthalpy than the reversible unfolding process, e.g. [37,38]. Similarly, Zoungrana et al. [24] assumed that the heat effect recorded by DSC is essentially due to the unfolding process.

For HSA dissolved in the supernatant after an adsorption experiment essentially the same thermal denaturation parameters were measured as for the native protein. Thus, the original structure does seem to be restored upon release from the solid surface if exchange of proteins in the adsorbed layer is assumed. As an example, Fig. 5 compares DSC thermograms of HSA in solution (PBS) before adsorption and after being released (by homomolecular exchange) from silica particles.

4. Discussion

4.1. Adsorption isotherms

It is generally accepted that the most important interaction forces between proteins and solids are hydrophobic interaction and electrostatic attraction and/or repulsion interactions, combined with an entropy gain caused by conformational changes of the protein during adsorption [39]. The thermodynamics of protein–surface interaction has been discussed extensively by Norde and co-workers [40–42]. They concluded that adsorption, or lack thereof, is primarily a reflection of the stability of the protein. While proteins and surfaces of opposite charge always interact adsorptively, they found the converse not to be universally true. The driving force for adsorption in this case is likely not enthalpic in origin, but rather of entropic nature and resulting, as far as it concerns the protein, from its gain in configurational entropy upon unfolding. Based on this considerations, Norde introduced the terminology of ''hard'' and ''soft'' proteins, respectively to describe those repelled by, and adsorbing to, hydrophilic surfaces of similar charge.

At pH 7.1/7.4, the silica surface is negatively charged and α-chymotrypsin is slightly positively charged (IEP (α-chymotrypsin) = 8.1), so that weak electrostatic interactions between the adsorbent and the protein could be a driving force for adsorption in the present study. HSA adsorbs spontaneously to the hydrophilic silica surface, although it is negatively charged at pH 7.4 (IEP (HSA) = 4.7). Thus, the possibility of decrease in ordered structure in these ''soft'' protein molecules favoring the adsorption process should not be overlooked [43]. This is consistent with the DSC data (see following sections).

According to the molecular dimensions of the native enzyme, a closed packed monolayer of α-chymotrypsin is calculated to be in the range 2.6–3.3 mg/m^2 depending whether the molecules are adsorbed side-on or end-on [24]. On the same basis, a closely packed monolayer of HSA corresponds to 1.9–7.6 mg/m^2. Thus, the plateau values of the adsorption isotherms of HSA indicate that completion of an adsorbed monolayer does not occur, irrespective of the orientation of the adsorbed molecules. This could be a hint for a greater area required by partially unfolded or fully denatured HSA (see DSC data). The maximum adsorption for α-chymotrypsin on silica (Aerosil OX-50 and Spherosil XOB 015 M) in 10 mM phosphate buffer and at low adsorbent content of the dispersion approaches monolayer coverage (side-on). The adsorption isotherm of α-chymotrypsin on silica (Aerosil OX-50) at $T = 20\,°C$, pH 7.1, $I = 0.01$ M given by Zoungrana et al. [24] agrees rather well with the adsorption isotherm we obtained in the present study for the same conditions with a low adsorbent/solution ratio (5 mg/ml) whereas the adsorption isotherm recorded for higher adsorbent/solution ratios (15 mg/ml) significantly differs from Zoungrana's results. A direct comparison of the data is difficult, as Zoungrana et al. did not mention the adsorbent/solution ratio they used.

At high adsorbent/solution ratio the adsorbed amount is largely reduced. A possible explanation

may be aggregation/bridging of the particles. Complete charge reversal of negative charged silica particles by adsorption of positive charged α-chymotrypsin may not be possible, before collision occurs. Bridging of the particles by protein molecules may be the consequence. At low adsorbent area the protein is able to reverse charge of the particles quick enough to prevent aggregation by electrostatic repulsion.

4.2. Desorption

The desorption results, presented in this study, agree with those of Norde and Favier [44] who measured no significant desorption for BSA adsorbed onto silica particles (Carb-O-Sil M5, Cabot Corp.) at pH 7.0 in 0.01 M phosphate buffer, even over a period of several days. Similarly, the adsorption of BSA on glass (borosilicate) powder dispersed on 0.05 M phosphate buffer at pH 7 and 25 °C is a non-equilibrium irreversible process [40]. However, MacRitchie found that the adsorption of BSA at hydrophilic silica at pH 7.5 is reversible upon dilution [45].

For α-chymotrypsin on silica particles (0.01 M phosphate buffer, pH 7.1), Zoungrana et al. [24] found no desorption in the low concentration domain of the isotherm, whereas in the present study desorption upon dilution was below the detectable limit, regardless of the protein surface concentration.

There appear to be two factors involved in the question of why desorption curves do not always follow the adsorption curves. Firstly, under conditions where interfacial coagulation occurs, a true adsorption isotherm is not obtained. Secondly, desorption under certain conditions may be an extremely slow process. Whereas, adsorption of a protein molecule apparently requires only a relatively small segment of the molecule to enter the interface [46], desorption by necessity requires that a large number of segments leave the interface together. Each of these segments may have an appreciably high free energy of adsorption so that the total free energy of the molecule may become very high [47].

4.3. Adsorption-induced structural changes—DSC data

Our calorimetric results confirm the findings of Haynes and Norde [43] that the tendency to undergo structural changes depends on the stability of the protein in the native-state. HSA retains virtually none of its compact native-state secondary structure upon adsorption to silica. The notion that HSA is a ''soft'' protein [40] is, therefore, fully supported by the calorimetric data. In contrast, α-chymotrypsin, which is a relatively stable globular protein, retains most of its native-state structure upon adsorption to silica. These findings were on the whole unaffected from the experimental conditions chosen in this study, although different experimental conditions resulted in different adsorption isotherms. Estimates of the conformational stability based on the Gibbs energy change at protein folding, which have been reported by other authors [48,49], corroborate the lower stability of HSA compared to that of α-chymotrypsin.

Differential scanning calorimetry is a useful method for assessing some important aspects of the conformational state of proteins when adsorbed to solid surfaces.

Several recent studies with DSC methods indicated that some adsorbed proteins may lose much of their structure. Haynes and Norde [43] observed that the transition enthalpy of lysozyme adsorbed to negatively charged polystyrene was much less than that for the protein in solution. Feng and Andrade [26] have shown that several proteins adsorbed to pyrolytic carbon no longer show any release of heat at the expected transition temperatures, suggesting that pyrolytic carbon induces complete unfolding, which is consistent with the tenacious binding of proteins to the surface. However, Yan et al. [23] have shown that albumin and lysozyme unfold extensively as they adapt to polystyrene surfaces. Steadman et al. [25] used micro-DSC to establish denaturation trends for seven globular proteins adsorbed to silica and to several chemically modified silica surfaces. Based on the T_d data, the stabilities of all seven proteins decreased upon adsorption to unmodified silica with the least stable native-state protein showing largest shift in T_d, and vice versa. For lysozyme, destabilization of the adsorbed-state structure was found to increase with increasing adsorbent-surface hydrophobicity. Billsten et al. [50] investigated conformational changes of human carbonic anhydrase II adsorbed to silica nanoparticles using DSC. To relate the observed conformational changes to the denaturation stability and/or chemical properties in solution, two N-terminally truncated variants and two mutants containing specific single site mutations were also investigated.

All these results indicate that changes in the enthalpy of unfolding depend on the adsorbing surface and the protein.

4.4. Comparison with literature data obtained by different methods

In the following, the results obtained in the present study should be compared with literature data dealing with structural changes of human or bovine albumin and α-chymotrypsin on hydrophilic surfaces.

Norde and Favier [44] measured the α-helix content of bovine serum albumin on finely dispersed silica particles. A decrease in α-helix content was observed upon adsorption, which increased with decreasing concentration of the protein in the bulk solution (i.e. decreasing surface coverage). Similar results were reported by Kondo et al. [13] for the adsorption of albumin on silica. van Wagenen et al. [51] found that albumin undergoes significant conformational changes upon adsorption to hydrophilic glass using fluorescence spectroscopy. Cuypers et al. [8,52] used ellipsometry to examine layers of albumin adsorbed to hydrophobic chromium and hydrophilic chromium oxide surfaces. From the layer thicknesses they concluded that albumin forms a compact, partially denatured layer on both the hydrophobic and the hydrophilic surface. Norde and Giacomelli [53] observed that BSA adsorbed on hydrophilic silica particles does not show a thermal transition up to 90 °C; the α-helix content is lowered at this surface (pH 7, 10 mM phosphate buffer).

Thus, the results presented in the present paper are in agreement with several direct and indirect observations from previous literature. However, the DSC data do not point to a dependence of the adsorbed structure on the surface-bound albumin amount.

Our findings that α-chymotrypsin behaves typically like a ''hard'' protein and undergoes no or only slight adsorption-induced structural changes agree with some previous studies in literature, too. Baron et al. [54] investigated the adsorption of α-chymotrypsin on montmorillonite by transmission FTIR spectroscopy. A comparison of the pH-dependent structural changes for the solution and adsorbed-states probes the electrostatic origin of the adsorption. In the pD range 4.5–10, adsorption only perturbs some peripheral domains of the protein compared to the solution. Secondary structure unfolding affects about 15–20 peptide units. However, the inactivation of the catalytic activity of the adsorbed enzyme in the 5–7(9) pD range is due less to these structural changes than to steric hindrance. Based on these findings the authors put α-chymotrypsin in the class of ''hard'' proteins in relation to the stability of proteins at solid/liquid interfaces. Zoungrana et al. studied the structure, stability and activity of α-chymotrypsin adsorbed onto different particles [24]. Contact with hydrophilic silica surfaces leads to a loss in helix content. However, DSC points to a heterogeneous population of adsorbed protein molecules with respect to their conformational states. In contrast to our results the authors found that the fraction of native-like conformation in the adsorbed layer increases with increasing coverage of the silica surface by the proteins. The specific enzymatic activity in the adsorbed-state quantitatively correlates with the fraction of proteins in native-like conformation.

4.5. Release from the adsorbed-state by homomolecular exchange

For hydrophilic surfaces like silica a homomolecular exchange is expected to take place (compare Experimental) whereas for hydrophobic surfaces the situation may be completely different. Assuming such an adsorption/desorption equilibrium a large fraction of the molecules in the supernatant have been in contact with the silica surface. The DSC data of the present study point out that the structure of adsorbed HSA is completely restored upon release from the solid surface. These observations differ from those made of Norde and Favier [44] who found that after displacement from silica surfaces the helical structure is regained, although not to the extent of that in the native molecule. However, they are in line with the results reported by Kondo and Fukada [55] by CD spectroscopy who reported that the percentage of α-helix content in adsorbed BSA on ultrafine silica particles decreased and that the desorbed proteins refolded to give structures similar to the native-state. Therefore, the structural changes of BSA upon adsorption onto ultrafine silica are reversible. Similar results were obtained by Norde and Giacomelli [53] using both DSC and CD: after release from hydrophilic silica the BSA molecule re-adopts the same conformational characteristics as before adsorption.

As α-chymotrypsin does not show measurable structural perturbations even in the adsorbed-state, one would not expect that α-chymotrypsin released from the solid substrate would exhibit structural alterations compared to the native protein before adsorption. Due to the low concentrations of α-chymotrypsin in the supernatants, determination of valuable thermograms was impossible in most cases.

5. Conclusions

The proteins HSA and α-chymotrypsin adsorb irreversibly from aqueous buffered solution onto hydrophilic silica surfaces. Calorimetric experiments clearly indicate severe structural rearrangements in HSA resulting from adsorption. α-chymotrypsin does not undergo significant conformational changes upon adsorption.

The results suggest that electrostatic interactions constitute the main mechanism involved in the adsorption of α-chymotrypsin onto silica, whereas adsorption of HSA on silica is mainly entropically driven and related to the adsorption-induced conformational changes (unfolding) of the protein.

Upon release from the adsorbed-state by homomolecular exchange the structure of adsorbed HSA is restored.

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