

Detailed Characterization of
Adsorption-Induced Protein Unfolding

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Detailed Characterization of Adsorption-Induced Protein Unfolding

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‘Weisheit ist nicht mitteilbar. Weisheit, welche ein Weiser mitzuteilen versucht, klingt immer wie Narrheit.’

‘Wisdom is not communicable. The wisdom, which a wise man tries to communicate, always sounds foolish.’

Hermann Hesse

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Preface

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1 Introduction

The adsorption of protein molecules on interfaces is a ubiquitous phenomenon in both natural and man-made systems. The process of protein adsorption is part of everyday life: proteins adsorb for example on contact lenses, on dishes, and on oil/water interfaces in food. Many food products consist of an emulsion stabilized by adsorbed proteins, for example cookies, soup, and ice cream. Protein adsorption is defined as the spontaneous adhesion of a thin layer of protein molecules to a surface. In this thesis, the adsorption of a monolayer of protein molecules, i.e. a layer with the thickness of one molecule, is investigated.

The process of protein adsorption has been the subject of a large number of studies because of its importance in many systems. Several researchers excellently reviewed the theory and knowledge of protein adsorption (Andrade and Hlady, 1986; Haynes and Norde, 1995; MacRitchie, 1972; Norde, 1986; Norde, 1999; Wahlgren and Arnebrant, 1991). It is well recognized that protein molecules undergo conformational changes upon adsorption on an interface. These adsorption-induced conformational changes of proteins are an essential part of the process of protein adsorption. However, detailed knowledge of these conformational changes is lacking and hampers the further understanding of protein adsorption. Such knowledge is required to control protein adsorption in a variety of applications.

This chapter explains why protein adsorption is such an important process, and why knowledge of adsorption-induced conformational changes of proteins is required. Furthermore, this chapter summarizes the current knowledge of adsorption-induced conformational changes of proteins adsorbed on an interface and it introduces the characteristics of the specific protein and surface selected for the study of protein adsorption. Finally, the objective of this thesis is stated, followed by a short introduction of the topic of each of its chapters.

1.1 Protein adsorption is ubiquitous

Protein molecules are amphoteric molecules, i.e. they contain both acidic and basic moieties, and they are amphiphilic molecules, i.e. they contain both hydrophilic and hydrophobic moieties. This variety in chemical character causes proteins to adsorb on a wide range of different surfaces. Hence, it is not surprising that protein adsorption occurs in a large number of natural and man-made systems: protein adsorption is ubiquitous.

The adsorption of proteins is an important process in food systems; for example, proteins that are adsorbed on an oil/water interface can stabilize an emulsion. The conformational changes that adsorbed protein molecules undergo affect their function and properties in food systems. However, the knowledge of these conformational changes, like the possible induction of a molten globule state, is limited. The complexity of the system, i.e. the presence of a water phase, an oil phase, and a protein at the interface, makes it difficult to study conformational changes on such interfaces.

Besides the presence of adsorbed proteins in food, there are many other examples of systems in which protein adsorption is an important process. Protein purification and protein analysis by chromatography are examples of man-made systems that rely on protein adsorption (Chase, 1994; Regnier, 1987a). The successful use of chromatography requires knowledge of the optimal conditions, i.e. which pH, ionic strength, and temperature to use, to control protein adsorption. During adsorption, the structure of the proteins involved, from the primary to the quaternary structural level, plays an important role since it determines whether and how a protein binds to a certain surface (Regnier, 1987b).

The field of nanotechnology, which has recently gained much interest, provides examples in which protein adsorption is of great importance. Proteins can be adsorbed in nanoscale arrays, 110 to 350 nm in size, on a flat surface by the technique of lithography (Lee, et al., 2002). Similar nanoscale patterns of adsorbed proteins can be obtained using the Langmuir-Blodgett technique (Moraille and Badia, 2002). Such patterned surfaces can be applied in proteomics type of research or in pharmaceutical screening processes. The development of a microfluidic chip that can adsorb proteins from solution and release them on command is another example that has potential in proteomics research (Huber, et al., 2003).

The adsorption of protein molecules on an interface can also be undesired, as the following examples show. Proteins adsorb on synthetic material that is implanted in the human body. This material, for instance a hip implant, is exposed to fluids in the human body that contain protein molecules that immediately start adsorbing on its surface (Andrade, et al., 1986). It is thought that conformational changes of these adsorbed protein molecules can indirectly cause adverse reactions of the body, like inflammation and thrombosis, after implantation of the foreign material (Balasubramanian, et al., 1999; Hu, et al., 2001). Another example of health-related undesired protein adsorption is the well-known fouling of contact lenses (Furness, et al., 1998). Daily cleaning of the contact lenses with a surfactant is needed to prevent the accumulation of adsorbed proteins and the subsequent harmful processes.

Recently, it has been suggested that amyloid fibril formation can be induced by the adsorption of amyloid proteins on an air/water interface or on a hydrophobic solid

surface (Giacomelli and Norde, 2003; Kowalewski and Holtzman, 1999; Schladitz, et al., 1999). Indications exist that amyloid formation can be enhanced by the interaction of amyloid forming peptides and their prefibrils with membranes (Müller, et al., 2001; Porat, et al., 2003). Amyloid formation is related to a number of human diseases, like Alzheimer's and Parkinson diseases, Creutzfeldt-Jakob disease, and type II diabetes (Dobson, 1999).

Protein adsorption is usually the first step in the fouling of surfaces that are exposed to fluids containing protein molecules. Examples of such fluids are not only body fluids, but also seawater and milk. The latter two play a role in the fouling of ships' hulls (Halperin and Leckband, 2000) and processing equipment in the dairy industry (Michalski, et al., 1997), respectively. Fouling causes problems that entail high expenses, and research is required to find solutions to avoid protein adsorption. Anti-fouling solutions involve amongst others the use of long flexible molecules attached to the surface to prevent protein adsorption (Morra, 2000).

An example of protein adsorption that is essential in humans is the protein statherin, which plays a role in the growth of bone tissue in the human body. This protein adsorbs with an acidic domain on the surface of hydroxyapatite (Gilbert, et al., 2000). The binding to and recognition of the hydroxyapatite surface is essential for the function of this protein. The study of the conformation of this protein in the adsorbed state is important to understand its function.

Nearly all of the research into protein adsorption finds its roots in the fields or applications discussed above. Consequently, different approaches to study protein adsorption exist. Usually, the chosen approach is inspired by the specific circumstances that play an important role in the field involved. For example, in chromatography it is essential that proteins can be removed from column material after a separation step. In contrast, in food related fields, the removal of protein molecules adsorbed on oil/water interfaces is usually not required and thus not extensively studied. The approach followed in this thesis is mainly inspired by the protein-folding field, because protein unfolding plays an important role during protein adsorption, as is shown below.

1.2 Adsorbed proteins can undergo conformational changes

Protein molecules spontaneously adsorb on an interface when the corresponding free energy for adsorption, ΔG_{ads} , is negative. It has been shown that the major contribution to a negative ΔG_{ads} originates from hydrophobic and electrostatic interactions. However, conformational changes also significantly contribute to decrease ΔG_{ads} , thereby providing an additional driving force for adsorption of the protein (Norde and Lyklema, 1991; Norde, et al., 1995; Norde, 1999). The first observations that indicated

conformational changes of adsorbed proteins were made over 50 years ago (MacRitchie, 1972). These observations were based on measurements of the enzymatic activity of adsorbed proteins and of the interfacial pressure of proteins on air/liquid or liquid/liquid interfaces. Knowledge of these conformational changes is essential to understand and control protein adsorption, and to further develop the theory of protein adsorption.

In this thesis, three spectroscopic techniques are used to obtain detailed information about the conformation of adsorbed proteins: Circular Dichroism (CD) spectroscopy, fluorescence spectroscopy, and Nuclear Magnetic Resonance (NMR) spectroscopy. These techniques are discussed in more detail below.

1.3 Circular Dichroism spectroscopy

Circular Dichroism (CD) spectroscopy is used to study the secondary and tertiary structure of proteins. The specific conformation of amide bonds in secondary structure elements results in a typical pattern of the far-UV CD spectra (Johnson, 1990). The amount of α -helix and β -sheet can be estimated after fitting the far-UV CD spectra to the corresponding spectra of proteins with known secondary structure (Greenfield, 1996). Far-UV CD spectroscopy is used to study protein folding, as it allows the easy detection of changes in secondary structure of proteins (Kelly and Price, 1997).

Changes in the tertiary structure of proteins can be investigated with near-UV CD spectroscopy. The specific conformation and environment of aromatic residues gives a characteristic near-UV CD spectrum in folded protein molecules (Strickland, 1974). Even small conformational changes, which affect the position of aromatic residues but which do not necessarily affect the secondary structure of proteins, can be observed with near-UV CD spectroscopy. Since the aromatic residues Trp and Tyr are also fluorescent, near-UV CD spectroscopy is complementary to the technique of intrinsic fluorescence spectroscopy.

CD spectroscopy has been used previously to study the conformation of adsorbed proteins. Usually, a colloidal suspension of small particles provides the interface for protein adsorption in CD spectroscopic studies of adsorbed proteins. If the suspended particles are small enough, light scattering is minimized and the presence of the suspension in the cuvette does not significantly disturb the corresponding CD spectra. The extent of scattering is proportional to the inverse of the wavelength to the fourth power (λ^{-4}), consequently, light scattering is most disturbing at low wavelengths. Preferably, the particles used should not significantly absorb in the UV band and should have a refractive index that is close to that of water (r.i. = 1.33). Materials that have been successfully used for adsorption studies are: silica (r.i. = 1.46) (Billsten, et al., 1997; Bower, et al., 1999; Giacomelli and Norde, 2001; Maste, et al., 1997; Norde and

Giacomelli, 2000; Tian, et al., 1998), Teflon (Caessens, et al., 1999; Maste, et al., 1997), polystyrene (r.i. = 1.59) (Haynes, et al., 1994; Haynes, et al., 1995; Kondo, et al., 1992; Norde, et al., 2000; Vermonden, et al., 2001), titanium oxide (Yongli, et al., 1999), silver iodide (Vermonden, et al., 2001), and poly(methyl methacrylate) (Baptista, et al., 2003).

Light scattering in CD spectroscopy due to the presence of adsorbent particles can be reduced by using the method of refractive index matching. This method involves the addition of a compound with a higher refractive index than water, e.g. glycerol, to the buffer solution in which the particles are suspended (Husband, et al., 2001). This reduces the difference in refractive index between the particles and the solution, and as a result the light scattering is reduced. However, the added compound should not affect the conformation and thermodynamic stability of the proteins involved.

Besides colloidal suspensions of adsorbent particles, solid macroscopic interfaces can be used for the study of the structure of adsorbed proteins by CD spectroscopy. A cuvette containing several parallel quartz slides, placed perpendicular to the light path, provides enough surface for protein molecules to adsorb, such that sufficient CD signal is obtained to detect the secondary structure of the adsorbed protein layer (McMillin and Walton, 1974; Smith and Clark, 1992; Vermeer and Norde, 2000). Only surfaces which are transparent for UV light can be used in this approach.

1.4 Fluorescence spectroscopy

The intrinsic fluorescence of protein molecules is very sensitive to changes in their tertiary structure. It informs about the local environment and dynamics of the fluorescent amino acid residues Trp, Tyr and Phe and of fluorescent cofactors like flavin, if present (Lakowicz, 1999). The position of the spectral maximum in the fluorescence spectrum, the fluorescence quantum yield, and the fluorescence lifetime are indicators for changes in the local environment of these fluorescent groups in the protein molecule. Fluorescence spectroscopy is not necessarily restricted to the fluorescence of intrinsic fluorophores. Specific residues on the protein can be labeled with a fluorescent group, and yield information about the environment of that group (Tan and Martic, 1990). In addition, fluorescent probes like ANS and thioflavin T inform about the structure of proteins, without covalent interaction between the fluorescent probes and the protein (Karlsson, et al., 2000).

Analogous to CD spectroscopy, fluorescence spectroscopy can be used in combination with a system in which suspended adsorbent particles provide an interface for protein adsorption (Clark, et al., 1994; Tan, et al., 1990; Walton and Maenpa, 1979). In contrast to CD, the wavelengths used are higher and the concentrations of proteins

and particles used are lower. This drastically reduces the effect of light scattering in fluorescence spectroscopy compared to CD spectroscopy. In addition, fluorescence spectroscopy is a more sensitive technique than CD spectroscopy.

Besides steady state fluorescence spectroscopy, also time-resolved fluorescence spectroscopy (Czeslik, 2001; Czeslik and Winter, 2001; Maste, et al., 1996), stopped-flow fluorescence, and fluorescence anisotropy (Maste, et al., 1996; Pap, et al., 1996; Tan, et al., 1990) can be used to investigate conformational changes of adsorbed protein molecules. Fluorescence anisotropy is directly related to the rotational correlation time of the fluorescent group involved (Lakowicz, 1999) and can be used to detect whether protein molecules are adsorbed. Adsorption of a protein molecule on a particle that is much larger than the protein itself increases the rotational correlation time of the adsorbed protein and thus increases its fluorescence anisotropy.

1.5 Nuclear Magnetic Resonance spectroscopy

The three-dimensional structure of protein molecules is generally obtained by the well-established methods of X-ray crystallography and NMR spectroscopy (Wüthrich, 1995). However, the suitability of these techniques for the study of adsorbed proteins is severely limited by experimental difficulties, mainly due to the presence of a solid adsorbent. X-ray crystallography requires protein crystals and thus is not suitable for the study of the structure of adsorbed proteins, unless these adsorbed proteins form a two-dimensional crystal, which is very difficult to achieve (Hasler, et al., 1998). NMR spectroscopy has been scarcely used to study the structure of adsorbed proteins. Limited information about the conformation of adsorbed proteins has been obtained by solid-state NMR spectroscopy (Drobny, et al., 2003; Mayer, 2002). The relaxation rates of solvent protons near adsorbed proteins has been studied in solution by NMR spectroscopy (Benko, et al., 1975). The conformation of small adsorbed peptides, composed of up to 14 amino acid residues, could be determined in solution by NMR spectroscopy (Burkett and Read, 2001).

Up to now, NMR spectroscopic studies in solution of protein molecules adsorbed on a solid interface have not been reported. This is not surprising since the motions of the required solid particles are on an undesirable time scale, which leads to severe line broadening of the NMR signal. However, the latter phenomenon can be favourably exploited as is shown in Chapter 5. NMR spectroscopists usually avoid samples that contain suspended particles since the expected inhomogeneity of these samples would cause magnetic field differences that lead to disturbed solution NMR spectra of proteins.

NMR spectroscopy can be used in combination with hydrogen/deuterium (H/D) exchange as a method to study in an indirect way the properties of previously adsorbed proteins after their removal from the interface. In this method, indirect information about the conformational properties of the adsorbed protein is obtained by allowing amide hydrogen atoms of the adsorbed protein molecules to exchange with deuterated solvent during a defined period. After displacement of the adsorbed protein molecules from the surface and quenching of this exchange, the exchange can be quantified in solution by two-dimensional ^1H -NMR spectroscopy, as shown in Chapter 5. The exchange characteristics can inform about the local stability of the adsorbed protein and about the parts of the protein that are solvent inaccessible due to adsorption.

In the few reported studies of adsorbed proteins by H/D exchange in combination with NMR spectroscopy, only limited information about the conformation and the local stability of the adsorbed protein could be obtained (Gorenstein, et al., 1994; Keire, et al., 1992; McNay, et al., 2001a; McNay, et al., 2001b). Exchange rates of only 4 protein residues (Nagadome, et al., 1993) or exchange information that is based on just a single point in time have been reported (Keire, et al., 1992; McNay and Fernandez, 1999). Mass spectrometry has been used with the intention to study H/D exchange of adsorbed proteins, but only the exchange behaviour of large peptide fragments could be characterized (Buijs, et al., 1999; Buijs, et al., 2000; Buijs, et al., 2003). In chapter 5 of this thesis, it is shown that NMR spectroscopy in combination with H/D exchange gives detailed information at the residue level about the orientation, conformation, and stability of a protein adsorbed on a solid interface.

1.6 The adsorption of bovine α -lactalbumin

This thesis reports the study of the conformational characteristics of the protein bovine α -lactalbumin (BLA) adsorbed on negatively charged, hydrophobic, polystyrene nanospheres. BLA is chosen to study protein adsorption for several reasons. Firstly, its structure, stability, and folding behaviour in solution have been thoroughly investigated (Chrysina, et al., 2000; Greene, et al., 1999; Kuwajima, 1996). Secondly, the interaction of BLA with a variety of interfaces has been studied with several techniques (see below). Finally, BLA is actually applied as a surface-active protein, for example in food applications.

Bovine α -lactalbumin (BLA) is a 14 kD protein present in milk. BLA itself is not an enzyme, but a specificity modifier of the enzyme galactosyltransferase (McKenzie and White, 1991). BLA binds to this enzyme, which results in an increase of the affinity of the BLA-galactosyltransferase complex for the substrate glucose. The three-dimensional structure of BLA has recently been determined by X-ray crystallography (Fig. 1-1)

(Chrysina, et al., 2000; Pike, et al., 1996). BLA consists of an α -domain, mainly containing α -helices, and a β -domain, containing a β -sheet. A calcium-binding site is located on the interface of these two domains. The calcium ion plays an important role in the stability and folding behaviour of the protein (Troullier, et al., 2000).

One of the most studied examples of a stable protein folding intermediate is the molten globule state of BLA. This state is characterized by disordered tertiary structure and native-like secondary structure. A protein in the molten globule state has increased exposure of hydrophobic groups compared to its native state. This exposure can be detected by hydrophobic fluorescent probes like 1-anilinonaphthalene-8-sulfonate (ANS) (Semisotnov, et al., 1991).



Fig. 1-1. Cartoon representation of the X-ray structure of holo-BLA (PDB-file 1F6S)(Chrysina, et al., 2000). The grey ball represents the calcium ion. The four Trp residues are shown in ball & stick representation.

BLA contains four tryptophan residues. Upon excitation, energy transfer occurs from both Trp26 and Trp104 to Trp60. In addition, the fluorescence of Trp60 is quenched by two nearby disulfide bonds (Cys73-Cys91 and Cys61-Cys77) (Arai and Kuwajima, 1996). Unfolding of BLA results in different positions of the tryptophan and cysteine residues compared to their original position in native BLA, leading to an increase in the fluorescence intensity. Unfolding also results in a red shift of the fluorescence maximum, indicating a more exposed environment of the Trp residues. A shift in the fluorescence emission maximum from 325 nm in the native state to 350 nm in the presence of 5.4 M guanidine hydrochloride indicates complete exposure of the Trp residues, resulting from the unfolding of BLA.

1.7 Polystyrene nanospheres as adsorbents

Many different adsorbents are used to study protein adsorption. These adsorbents vary in charge, hydrophobicity and the presence of specific groups on their surface. In

the study presented in this thesis, two different polystyrene nanospheres, with diameters of 47 and 120 nm, are used to adsorb BLA molecules. BLA adsorbs with high affinity on these hydrophobic polystyrene nanospheres, as inferred from the steep rise of the adsorption isotherm at low BLA concentrations (see Chapter 2). At these BLA concentrations, all protein present in the system is adsorbed on the surface of the polystyrene nanospheres and virtually no free BLA is present in solution. This situation is advantageous for spectroscopic studies as all spectroscopic signals observed originate from adsorbed protein molecules and no contributions from free protein molecules need to be taken into account.

The large surface area per gram of polystyrene nanospheres and therefore the high binding capacity allows the use of low nanosphere concentrations. As a result, light scattering and light absorption can be minimised, which makes the use of spectroscopic methods like stopped-flow fluorescence feasible. Although the nanospheres used in this thesis are not a biological system, nor an oil/water interface as present in an emulsion, they are, at the moment, the only interface system that allows the investigation of the effect adsorption has on protein conformation using kinetic spectroscopic methods (Chapter 2).

The two different polystyrene nanospheres used in the study presented here are characterized by several methods. Dynamic light scattering shows that the diameters of the two different nanospheres used are 47 and 120 nm, respectively. The corresponding surface areas are 122 and 48 m²/g, respectively. The nanospheres have a slightly negative surface charge, mainly due to the presence of sulphate groups on their surface. Investigation by transmission electron microscopy (TEM) shows that the nanospheres are spherical and that possible irregularities at the surface are smaller than 2 nm. The polystyrene nanospheres do not aggregate after BLA adsorbs on them. However, aggregation can occur if the salt concentration is too high.

1.8 Indications for adsorption-induced conformational changes of BLA

Many experimental studies show that the conformation of adsorbed proteins differs from their native conformation. This is also true for BLA adsorbed on a variety of solid surfaces. Calorimetric measurements indicate that adsorption of BLA on hydrophobic surfaces is partly driven by increased conformational entropy due to structural rearrangements (Arai and Norde, 1990b; a). Isothermal titration calorimetry (ITC) and proton titration of BLA adsorbed on polystyrene spheres indicate substantial changes in the secondary and tertiary structure of BLA compared to native BLA (Haynes, et al., 1994; Haynes, et al., 1995). Conformational changes of BLA adsorbed on Teflon are shown to occur from measurements of the interfacial tension (van der Vegt, et al.,

1996a; b). The release of calcium from holo-BLA after its adsorption on a hydrophobic polystyrene surface is inferred from the similar adsorption behaviour of apo- and holo-BLA, and the similar proton titration behaviour of their adsorbed states (Galisteo and Norde, 1995a; b). Changes in the fluorescence intensity and corresponding spectral maximum show that apo-BLA unfolds on hydrophobic chromatographic surfaces (Oroszlan, et al., 1990).

Hydrophilic surfaces are thought to be less denaturing than hydrophobic surfaces. Using Raman spectroscopy, it is shown that the secondary structure of BLA does not significantly change upon its adsorption on S-Sepharose, a hydrophilic agarose based cation-exchange material (Sane, et al., 1999). The absence of significant changes in the conformation of holo-BLA adsorbed on hydrophilic silica particles is also observed with DSC measurements (Larsericdotter, et al., 2001). From the latter study, it is concluded that calcium is still bound to BLA in the adsorbed state. However, ATR-FTIR measurements of the adsorption of apo-BLA on a hydrophilic germanium surface show the loss of secondary structure and a concomitant increase in unordered structure of the adsorbed protein (Bentaleb, et al., 1999).

Oil/water interfaces are generally more denaturing than solid interfaces. The increase in β -sheet structure at the expense of α -helical structure is observed for BLA adsorbed on a soy bean oil/water interface (Fang and Dalglish, 1998). Compared to native BLA, the molten globule state of BLA adsorbs preferentially on an oil/water interface as shown by competitive adsorption experiments (Matsumura, et al., 1994). The molten-globule state of globular proteins is suspected to be relevant during adsorption on oil/water interfaces (Dickinson and Matsumura, 1994; Hirose, 1993).

Besides on solid/liquid and liquid/liquid interfaces, proteins also adsorb on lipid bilayers and insert in them. The interaction of BLA with lipid vesicles, and the influence of different BLA conformations on this interaction, was studied in the early 1980's (Hanssens, et al., 1979; Hanssens, et al., 1980; Hanssens, et al., 1983; Hanssens, et al., 1985; Herreman, et al., 1981). It was found that interaction with these vesicles is increased upon unfolding of the tertiary structure of BLA. This work has resulted in further investigations of the binding of BLA to lipid bilayers (Berliner and Koga, 1987; Kim and Kim, 1989). Binding of BLA to lipid bilayers is shown to result in a molten globule-like conformation of BLA (Agasøster, et al., 2003; Bañuelos and Muga, 1996a; b; Cawthorn, et al., 1996; Halskau, et al., 2002).

In conclusion, there is considerable evidence that structural changes occur in BLA upon its adsorption on solid hydrophobic interfaces. However, the detailed characteristics of these adsorption-induced conformational changes are largely unknown, and many questions remain. How much of the tertiary or secondary structure is lost upon protein adsorption? Where in the adsorbed protein molecule do the

conformational changes occur? Does the (local) stability of the adsorbed protein change compared to the native protein in solution? What are the kinetics of the adsorption-induced conformational changes? Is the conformation of each adsorbed protein molecule affected in the same way? It is clear that further knowledge of adsorption-induced protein unfolding is needed to fully understand the process of protein adsorption. This thesis aims to answer some of these questions.

1.9 Objective of this thesis

The objective of the study presented here is to obtain detailed information about the conformational changes that occur in bovine α -lactalbumin (BLA) upon its adsorption on negatively charged, hydrophobic polystyrene nanospheres. This information is obtained by the use of three spectroscopic techniques: fluorescence spectroscopy, Circular Dichroism (CD) spectroscopy, and Nuclear Magnetic Resonance (NMR) spectroscopy in combination with hydrogen/deuterium (H/D) exchange. In addition, the kinetics associated with adsorption-induced conformational changes are obtained by stopped-flow fluorescence spectroscopy.

1.10 Outline of the thesis

The structural characterization of BLA adsorbed on polystyrene nanospheres, as determined by CD and fluorescence spectroscopy, is presented in Chapter 2. In addition, the rate of BLA adsorption on these nanospheres and the rate of the corresponding adsorption-induced conformational changes as determined by stopped-flow fluorescence spectroscopy are reported in this chapter. The results show that partial unfolding of BLA induced by its adsorption on a hydrophobic interface is surprisingly fast. Chapter 3 reports a procedure for the efficient displacement of adsorbed BLA by the addition of a surfactant. The displaced BLA molecules show native-like CD and fluorescence spectral properties, indicating that the adsorption-induced conformational changes are reversible upon displacement of adsorbed BLA by a surfactant. The complete refolding at the atomic level of displaced BLA in solution to native BLA is confirmed in Chapter 4 by two-dimensional ^1H -NMR spectroscopy. In addition, it is shown that NMR spectroscopy can be used to study BLA in the presence of BLA- or CHAPS-covered solid polystyrene nanospheres, without the nanospheres significantly affecting the quality of the NMR spectra. The results presented in Chapters 3 and 4 are the basis of the work presented in Chapter 5. The latter chapter describes the detailed study by H/D exchange and two-dimensional ^1H -NMR spectroscopy of the conformation, orientation and stability of BLA adsorbed on suspended polystyrene nanospheres. It is shown at the residue-level that BLA adsorbed on hydrophobic

polystyrene nanospheres has a conformation similar to the acid-denatured state (A-state) of BLA. Furthermore, it is proposed that the adsorption of proteins on hydrophobic interfaces can be a valuable tool for the study of protein folding intermediates. Finally, the main results of this thesis and their implications for the future study of adsorbed proteins are summarized.

2 Kinetic and structural characterization of adsorption-induced unfolding of bovine α -lactalbumin

Conformational changes of bovine α -lactalbumin induced by adsorption on a hydrophobic interface are studied by fluorescence and circular dichroism spectroscopy. Adsorption of bovine α -lactalbumin on hydrophobic polystyrene nanospheres induces a non-native state of the protein, which is characterized by preserved secondary structure, lost tertiary structure and by release of calcium. This partially denatured state therefore resembles a molten globule state, which is an intermediate in the folding of bovine α -lactalbumin. Stopped-flow fluorescence spectroscopy reveals two kinetic phases during adsorption with rate constants $k_1 \sim 50 \text{ s}^{-1}$ and $k_2 \sim 8 \text{ s}^{-1}$. The rate of partial unfolding is remarkably fast and even faster than unfolding induced by the addition of 5.4 M guanidine hydrochloride to native α -lactalbumin. The high unfolding rates exclude the possibility that unfolding of bovine α -lactalbumin to the intermediate state occurs before adsorption takes place. Stopped-flow fluorescence anisotropy experiments show that adsorption of bovine α -lactalbumin on polystyrene nanospheres occurs within the dead time (15 ms) of the experiment. This shows that the kinetic processes as determined by stopped-flow fluorescence spectroscopy are not affected by diffusion or association processes, but are solely caused by unfolding of bovine α -lactalbumin induced by adsorption on the polystyrene surface. A scheme is presented that incorporates the results obtained and describes the adsorption of bovine α -lactalbumin.

2.1 Introduction

Adsorption of proteins on solid/liquid interfaces is a generally occurring phenomenon both in nature and in man-made systems. It is well recognised that proteins undergo conformational changes upon adsorption on solid/liquid interfaces (Andrade, et al., 1986; Haynes, et al., 1995; Norde, 1999). Since unfolding of proteins can have a large effect on their function or properties, it is important to increase the knowledge about protein adsorption and about the resulting conformational changes. It is necessary to study not only the conformation of a protein in the adsorbed state, but also the kinetics of the adsorption-induced conformational changes. This knowledge will help in controlling wanted or unwanted conformational changes of adsorbed proteins.

This work focuses on the kinetics of adsorption-induced conformational changes of bovine α -lactalbumin (BLA) upon interaction with colloidal polystyrene nanospheres. Detailed information on adsorption-induced conformational changes and especially on the kinetics of adsorption-induced conformational changes is sparse. An important reason for this is the experimental difficulty of the presence of an adsorbent, which is usually a solid phase. A few routes have been designed to tackle this difficulty. A macroscopic solid/water interface can be used in combination with a reflective technique like ellipsometry or total internal reflection fluorescence (TIRF) (van Wagenen, et al., 1982; Wahlgren, et al., 1993). In other cases a microscopic system has been used which consists of small particles with diameters ranging from nanometers to micrometers (Maste, et al., 1996; Norde and Favier, 1992b). The latter system has the advantage of providing a large interface, however, light scattering and light absorbance often interfere with several spectroscopic techniques. Here, a suspension of polystyrene nanospheres with a diameter of approximately 50 nm is used. The large surface area per gram of polystyrene and therefore the high binding capacity allows a reduction of the nanosphere concentration. As a result, light scattering and absorption are reduced and thus the use of spectroscopic methods like stopped-flow fluorescence are feasible.

Bovine α -lactalbumin is a small protein (14 kDa), containing four tryptophan residues and four disulphide bonds. It can bind calcium, which plays an important role in the stability and folding behaviour of the protein (Troullier, et al., 2000). BLA is chosen in this study for several reasons. First, the structure, stability and folding behaviour of BLA have been thoroughly studied (Chrysina, et al., 2000; Greene, et al., 1999; Kuwajima, et al., 1985). Secondly, BLA is a protein that is applied at solid/water interfaces, for example in food applications. Finally, the folding pathway of BLA includes a stable intermediate, the so called molten globule state, which has been

studied in detail (Kim, et al., 1999; Kuwajima, 1996; Vanderheeren and Hanssens, 1994).

Isothermal titration calorimetry and intrinsic fluorescence spectroscopy have indicated denaturation of BLA upon adsorption on hydrophobic interfaces (Haynes, et al., 1994; Oroszlan, et al., 1990). A study of Bañuelos shows that BLA binds to lipid bilayers in a molten globule-like conformation (Bañuelos and Muga, 1995). Recently some studies emerged that describe the kinetics of conformational changes induced by adsorption on an interface or interaction with lipid vesicles (Karlsson, et al., 2000; Sanghera and Pinheiro, 2000; Surrey and Jähnig, 1995). Unfortunately, still little is known about the altered conformation in the adsorbed state, and about the kinetics of the conformational changes that take place upon adsorption. Further knowledge on these matters is of particular relevance for the understanding of protein-membrane interactions and in the field of synthetic biomaterials used for medical applications.

Regarding protein-membrane interactions, it is hypothesised that when proteins are translocated across the membrane, they must be in a non-native or molten globule (MG) state (Ptitsyn, 1995). Previous work has indicated that upon interaction with model membranes, BLA indeed can adopt an MG state (Bañuelos, et al., 1995). However, the interaction of proteins with model membranes can occur via two mechanisms: (a) adsorption on the membrane interface and (b) combined adsorption and insertion/penetration into the membrane bilayer (Hanssens, et al., 1980). In the study presented here, BLA is adsorbed on a solid interface, so insertion or penetration cannot occur. The results show that adsorption itself suffices to cause a conformational change of BLA to an adsorbed state with molten globule properties.

Although the polystyrene nanospheres used are not a biological system, they are, at the moment, the only solid/liquid interface system that allows the study of the effect of adsorption (excluding penetration) on protein conformation with kinetic spectroscopic methods. The results obtained widen the biological view of protein-membrane interactions.

Furthermore, this study is relevant in the field of synthetic biomaterials. Synthetic polymer biomaterials are used in medical applications, for example as implants in the human body. Contact between these biomaterials and tissue or blood gives rise to all kinds of complex and mostly unwanted phenomena. Upon implantation of foreign material in the human body, proteins adsorb on the biomaterial. Knowledge of this process is relevant in the search for new and better biomaterials that can reduce adverse reactions of the human body.

In this chapter, the adsorption-induced unfolding of bovine α -lactalbumin (BLA) is studied by intrinsic fluorescence and circular dichroism (CD) spectroscopy. Both the nature and the kinetics of conformational changes of BLA upon adsorption on a

polystyrene/water interface are characterized. Several theoretical models exist that describe the kinetics of adsorption as well as the kinetics of adsorption-induced conformational changes (Andrade, et al., 1986). The experimental difficulty of discriminating between both kinetic processes has hampered the experimental verification of these models. Here it is shown that the kinetics of adsorption and the kinetics of adsorption-induced conformational changes can be distinguished. In fact, it is demonstrated that the kinetics of both the diffusion and the adsorption processes do not need to be taken into account to enable analysis of the kinetics of the conformational change of the adsorbed protein as observed in stopped-flow experiments.

2.2 Materials and methods

2.2.1 Materials

Bovine α -lactalbumin (Sigma L-5385) was used without further purification. Polystyrene nanospheres, supplied as a colloidal suspension in water, were obtained from Polymer Laboratories (Heerlen, The Netherlands). Nanosphere suspensions were diluted with buffer before use. All experiments were done in a 10 mM Tris/HCl buffer of pH 7.50 containing 1 mM CaCl_2 . The added calcium ensures a constant calcium concentration during the adsorption and unfolding experiments, since calcium has a drastic effect on the stability and folding behaviour of BLA. Nanopure water was used in all experiments (Sybron Barnstead NANOpure II).

2.2.2 Dynamic light scattering

Dynamic light scattering was performed on an experimental setup composed of a Lexal 150 mW laser operating at a wavelength of 514.5 nm, a photomultiplier and a personal computer containing an ALV5000 correlator card. Scattered intensities were recorded at a 90° scattering angle and at 20°C . A total of 10 measurements of each 10 seconds were averaged. The data were analysed by the ALV5000 software. Samples were diluted in the buffer mentioned above. The nanosphere concentration ranged from 0.05 to 0.25 mg/ml.

2.2.3 Electrophoretic mobility

The electrophoretic mobility of the polystyrene nanospheres (3 mg/ml) suspended in a 10 mM Tris/HCl buffer at pH 7.5 with 1 mM CaCl_2 was determined with a Malvern Zetasizer III. The Malvern computer program was used to calculate the zeta-potential from the electrophoretic mobility.

2.2.4 Adsorption isotherm

The adsorption isotherm was determined according to the depletion method. The amount of free protein after adsorption was measured spectrophotometrically using a molar extinction coefficient of $28,540 \text{ M}^{-1}\text{cm}^{-1}$ for BLA at 280 nm. The polystyrene nanospheres were separated from free α -lactalbumin by using Centricon 100 ultrafiltration devices (Millipore Corporation). Protein adsorption on the filter was checked and found to be negligible. The filter retained more than 99.8% of the polystyrene nanospheres. In a typical adsorption experiment 2.5 ml of protein solution was added to 2.5 ml of nanosphere suspension in a 10 ml polysulfone centrifuge tube (Nalgene, Oak Ridge). The tubes were then placed on a rocking table, and gently shaken at room temperature. Adsorption isotherms were determined after 30 minutes of adsorption and after 24 hours of adsorption. Different protein concentrations were used to construct an adsorption isotherm. The resulting adsorption isotherm was used to determine the molar protein to nanosphere ratio (n) that gave maximal protein monolayer adsorption and minimal free protein. This ratio was used in all adsorption experiments, unless mentioned otherwise. For determination of the desorbed amount of BLA, the BLA-covered nanospheres were separated from the solution with Centricon 100 ultrafiltration devices. Separation was done at $4 \text{ }^\circ\text{C}$ without calcium or at $20 \text{ }^\circ\text{C}$ and in the presence of either 10 mM or 100 mM CaCl_2 . Subsequently the concentration of BLA in solution was determined spectrophotometrically.

2.2.5 Determination of the K_d for calcium binding on the nanospheres

Polystyrene nanospheres (80 nM) were incubated with CaCl_2 at different concentrations (0, 5, 10, 40, 100, 200 and 400 μM) for 20 hrs at room temperature in a calcium free 10 mM Tris/HCl buffer at pH 7.5. The nanospheres covered with calcium were separated from the calcium containing solution by ultrafiltration (Amicon, YM50 membrane, Millipore Corporation). The filtrate was analysed for calcium content by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES). The amount of adsorbed calcium was calculated from the original amount and the amount of free calcium after filtration. An adsorption isotherm was constructed and fitted with a Langmuir isotherm: $[\text{Ca}]_{\text{adsorbed}} = (\text{B} * [\text{Ca}]_{\text{free}}) / (\text{K}_d + [\text{Ca}]_{\text{free}})$. In this equation, B is the plateau value of adsorption, and K_d is the dissociation constant. The concentration of CaCl_2 stock solutions was determined with ICP-OES.

2.2.6 Fluorescence spectroscopy

Fluorescence spectra and time-dependent fluorescence traces were measured in a quartz cuvette (10 * 4 mm) on a Fluorolog 2 (SPEX). The temperature of the cuvette holder was maintained at $20 \text{ }^\circ\text{C}$ with a water bath in all experiments. Excitation was at

300 nm, with excitation slits at 3 nm and emission slits at 5 nm. A blank, containing all components except protein, was subtracted from each sample. Nanosphere concentrations ranged up to 0.05 mg/ml. The total absorbance of the samples at 300 nm was kept below 0.1 to minimise the inner filter effect. To ensure a molar protein to nanosphere ratio of 500, the maximum protein concentration was about 0.5 μ M. In the manual mixing adsorption experiments, the nanospheres were added to the stirred protein solution with an automatic pipette. Typically, an amount of 100 μ l nanosphere suspension was added to 1.5 ml of protein solution. In time-dependent fluorescence measurements, care was taken to avoid photobleaching of the fluorophore, by ensuring discontinuous illumination with enough (dark) time between data points.

2.2.7 *Fluorescence anisotropy*

Fluorescence anisotropy was measured on a home-built fluorimeter equipped with two photomultipliers arranged in T-format (Thorn EMI 9863QA/350, operating in photon-counting detection mode). The light was generated by a 150 W short arc Xenon lamp and the excitation wavelength of 300 nm was selected in a monochromator (Bausch and Lomb) with a bandpass of 3.2 nm. Polarizers were used in both the excitation light path (rotatable Glan Taylor polariser) and the emission light path (Polaroid, sheet). The emission light was selected with a 335-nm cut off filter and a UG1 filter (Schott). A blank measurement, containing all components except BLA, was subtracted from each sample, and five measurements were averaged for each sample.

2.2.8 *Stopped-flow fluorescence spectroscopy*

Stopped-flow fluorescence and stopped-flow fluorescence anisotropy were measured on a BioLogic SFM4 equipped with a 2*2 mm cuvette (FC-20). Excitation was at 300 nm, excitation slits were set at 0.5 mm, resulting in a bandpass of 4 nm. A cut off filter of 335 nm (335FG01-25, Andover Corporation) and a 330wb60 bandpass filter (XF3000-25, Omega Optical Inc.) were used together to select the emission light. The temperature was kept constant at 20 °C with a thermostatic water bath. Ten measurements were averaged for each sample. In a typical run 150 μ l of a 5 μ M protein solution was mixed with 150 μ l of a 10 nM nanosphere suspension in 75 ms. Flow speeds of 4 ml/s resulted in a dead time of 15 ms. Faster flow speeds were not used because this resulted in signal distortion and bad reproducibility, probably due to cavitation. Results from the stopped-flow fluorescence experiments were analysed and fitted with the Padé-Laplace algorithm in the Bio-Kine software (Bio-Logic), using a minimum number of exponential phases. Prior to fitting the data, the signal of the polystyrene nanospheres was subtracted from the sample, and the time-axis was adjusted, resulting in a shift of the first data point to $t = 0$ s. A fit was evaluated and

judged to be correct when random noise around a horizontal line centred at zero was observed for the residual values. Stopped-flow fluorescence anisotropy was measured with a single photomultiplier and no polariser in the emission light path. A similar set up has been reported recently in a study on protein folding in solution (Canet, et al., 2001). In the anisotropy set-up the excitation light is modulated with a photo-elastic modulator to give alternating horizontally and vertically polarised light with a frequency of 100 kHz. The synchronised electronics connected to the photomultiplier simultaneously record the fluorescence emission intensity at vertical excitation (I_v) and at horizontal excitation (I_h). A blank, containing all components except BLA, was subtracted from the sample resulting in the corrected values I_v^* and I_h^* . The anisotropy (A) for each data point was then calculated in a spreadsheet application according to the equation: $A = (I_v^* - I_h^*) / (I_v^* + I_h^* / 2)$.

2.2.9 Circular Dichroism spectroscopy

CD measurements were performed on a Jasco J-715 spectropolarimeter, equipped with a Peltier temperature control system set at 20 °C. Calibration was performed with an ammonium d-10-camphorsulfonate solution in nanopure water of which the concentration was checked spectrophotometrically. Typical protein concentrations used were 1.5 μM in a 0.1 cm cuvette for far-UV CD and 4.5 μM in a 1 cm cuvette for near-UV CD measurements. Eight scans were averaged for each sample. The CD spectra of BLA adsorbed on the nanospheres were averaged from 160 and 128 scans for the far-UV region and near-UV region, respectively. In case of adsorbed BLA, sample and blank spectra were recorded alternately in sets of 32 scans, in order to avoid disturbances due to instrumental drift. For each set of 32 scans a fresh sample of BLA adsorbed on the nanospheres was used. The response time was 1 second, and the spectral bandwidth was 1.0 nm. Before analysis of the spectra, a blank, containing all components except BLA, was subtracted from the sample.

2.3 RESULTS

2.3.1 Characterization of the polystyrene nanospheres

The radius of the nanospheres diluted in a 10 mM Tris/HCl buffer at pH 7.50 with 1 mM CaCl_2 is 47 ± 1 nm as determined by dynamic light scattering. Based on this value a surface area of 122 ± 3 m^2/g is calculated. Investigation by transmission electron microscopy shows that the nanospheres are spherical and that possible irregularities at the surface are smaller than 2 nm in size (results not shown). The zeta-potential for the nanospheres as determined by electrophoretic mobility is -38 ± 1 mV in a 10 mM Tris/HCl buffer with 1 mM CaCl_2 at pH 7.5. This value indicates a negative surface

charge of the polystyrene nanospheres in this particular buffer. The diameter of the nanospheres after adsorption of a monolayer of BLA is 49 ± 1 nm and remains the same during 24 hours. No aggregation of the nanospheres occurs after adsorption of BLA.

2.3.2 Adsorption isotherm

The adsorption isotherm of BLA on polystyrene nanospheres is shown in Fig. 2-1. The plateau value of 3 ± 0.2 mg/m^2 agrees well with previously reported values. Adsorption of BLA on a Pt surface resulted in a value of 2.9 mg/m^2 (Cabilio, et al., 2000) and a value of 3.3 mg/m^2 was found for adsorption of BLA on a hydrophobic silicon surface (Suttiprasit, et al., 1992). The steep part in the region of low BLA concentration is an indication for high affinity behaviour. In the initial, steep part of the adsorption isotherm, all protein present in the system is adsorbed on the surface of the polystyrene nanospheres and virtually no free BLA is present in solution. If a molar nanosphere to protein ratio of 1 to 500 is chosen, then all BLA added is adsorbed and there is no free BLA present in the solution. This ratio corresponds with the region in the adsorption isotherm of the initial steep part, before a coverage of 3 mg/m^2 is reached. This ratio is used in the adsorption experiments presented below, assuming that all added protein is adsorbed on the nanospheres.

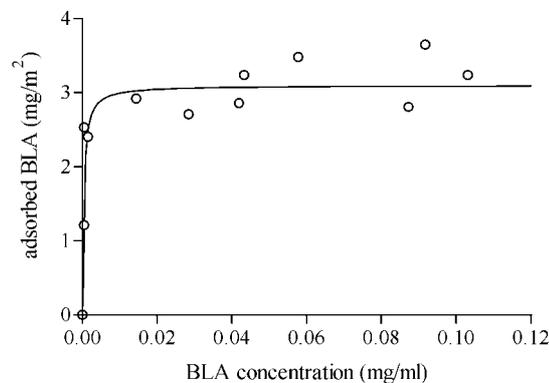


Fig. 2-1. Adsorption isotherm of bovine α -lactalbumin on polystyrene nanospheres (6.8 nM) in a 10 mM Tris/HCl buffer with 1 mM CaCl_2 at pH 7.5 after 24 hours of adsorption. Adsorption isotherms determined after an adsorption time of 30 minutes and after an adsorption time of 24 hours are identical.

The polystyrene nanospheres and the BLA molecules both have a net negative charge under the conditions used. The fact that adsorption occurs means that the electrostatic repulsion has to be overcome. Hydrophobic interactions therefore contribute the most to the adsorption process, as was found for the adsorption of BLA on hydrophobic interfaces (Haynes, et al., 1994; Larsericsdotter, et al., 2001). However, minor contributions of electrostatic interactions can not be excluded. They may play a role in the orientation of the adsorbed BLA molecules (see discussion section).

Desorption of BLA from the polystyrene nanospheres is tested with different methods. Washing the BLA covered nanospheres with buffer does not result in protein desorption, even after 24 hours. Hardly any desorption of BLA from the polystyrene nanospheres takes place when the calcium concentration is increased. Only 12% of the adsorbed BLA molecules is desorbed when the calcium concentration is increased to 10 mM, while only 14% is desorbed at a calcium concentration of 100 mM after 24 hours of incubation. In an attempt to decrease the hydrophobic interactions and enable BLA desorption the temperature was decreased. However, storage during 24 hours at 4 °C and in the presence of 1 mM CaCl₂ gave no desorption of BLA molecules.

2.3.3 Adsorption-induced conformational changes of BLA

Fluorescence spectra of BLA in solution and of BLA adsorbed on nanospheres after 5 and 75 minutes of adsorption are shown in Fig. 2-2. The spectrum of BLA in solution shows a fluorescence emission maximum at 325 nm which indicates that the Trp residues are buried in the hydrophobic interior of the protein. The adsorbed BLA shows a maximum at 335 nm and an intensity increase at maximum wavelength of about 100% compared to the spectrum of BLA in solution. The shift of the fluorescence maximum indicates that the tryptophan residues of BLA in the adsorbed state are in a more exposed environment. The intensity increase is related to a reduction in quenching that results from a different position of the tryptophan residues compared to their original position in the three dimensional structure of native BLA. For native BLA it is suggested that energy transfer occurs from Trp26 and Trp104 to Trp60 and, furthermore, the fluorescence is quenched by two disulfide bonds near Trp60 (Cys73-Cys91 and Cys61-Cys77) (Arai, et al., 1996). It has also been observed that unfolding of BLA results in a red shift of the fluorescence emission maximum and an accompanied increase in fluorescence intensity (Ostrovsky, et al., 1988). Complete unfolding of α -lactalbumin by 5.4 M guanidine hydrochloride results in a fluorescence emission maximum of 350 nm indicating complete exposure of the Trp residues (results not shown). A measured fluorescence emission maximum of 335 nm would thus indicate that the adsorption-induced unfolding is not complete, leaving one or more Trp residues in the hydrophobic core of BLA.

Circular dichroism spectra of free and of adsorbed BLA in the far-UV region and in the near-UV region are shown in Fig. 2-3. The far-UV CD spectrum of adsorbed BLA shows that a considerable amount of secondary structure remains after adsorption. Although the spectrum of adsorbed BLA contains a lot of noise, the characteristic ellipticity minimum at 208 nm is visible. This indicates that the secondary structure of native BLA and of adsorbed BLA is similar. The near-UV CD spectra show a clear difference between native BLA and adsorbed BLA. The spectrum of BLA in solution

has a characteristic profile which includes a broad Tyr minimum around 270 nm and a local Trp minimum at 297 nm. The spectrum of adsorbed BLA does not show these characteristics and has a decreased molar ellipticity over the entire near-UV CD spectrum, which indicates disruption of tertiary structure elements. The latter is in agreement with the changes seen in the fluorescence emission spectrum upon protein adsorption (see above).

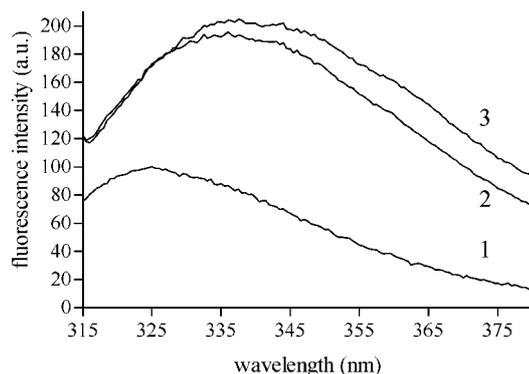


Fig. 2-2. Fluorescence emission spectra of 0.5 μM BLA in solution (1) and of 0.5 μM BLA adsorbed on 0.05 mg/ml polystyrene nanospheres after 5 (2) and 75 (3) minutes of adsorption in a 10 mM Tris/HCl buffer with 1 mM CaCl_2 at pH 7.5 and at 20 $^\circ\text{C}$. The fluorescence intensity is normalized to a value of 100 for the maximum at 325 nm for BLA in solution. The excitation wavelength was 300 nm.

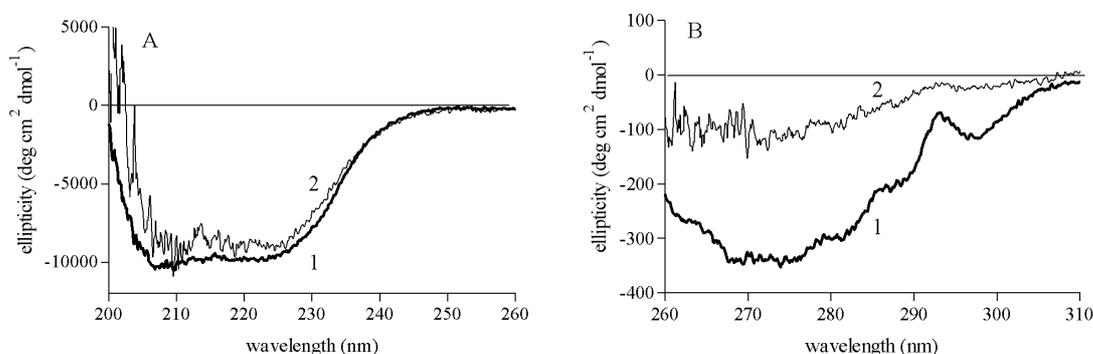


Fig. 2-3. Far-UV (A) and near-UV (B) CD spectrum of BLA in solution (1, thick line) and of BLA adsorbed on polystyrene nanospheres (2, thin line) in a 10 mM Tris/HCl buffer with 1 mM CaCl_2 at pH 7.5 and at 20 $^\circ\text{C}$. The concentrations used for far-UV CD spectra are: 1.5 μM BLA and 3 nM nanospheres, in a 1 mm cuvette. The concentrations used for near-UV CD spectra are: 4.5 μM BLA and 10 nM nanospheres, in a 10 mm cuvette.

The difficulty of working with polystyrene nanospheres in suspensions emerges in particular when working with far-UV light in CD spectroscopy (Fig. 2-3A). The CD measurements in the far-UV region were performed with a nanosphere concentration that results in a UV absorption of 0.5 at 220 nm in a 0.1 cm light path cuvette, while in

the near-UV a concentration of nanospheres is used which results in a UV absorption of 0.5 at 280 nm in a 1 cm light path cuvette. The protein concentration was adjusted to obtain a ratio of 500 protein molecules to 1 nanosphere particle, as is used throughout this work. The loss of light intensity in the CD measurements of the adsorbed protein, which is due to both light scattering of the polystyrene nanospheres and light absorption of the polystyrene molecules in the nanospheres, leaves only half of the original intensity for the characterization of the protein structure. Consequently, a significant increase in noise on the data is observed. Despite the difference in the noise level between the spectra shown in Fig. 2-3, it is clear that upon BLA adsorption a disruption of tertiary structure elements occurs without significant loss of secondary structure.

2.3.4 Kinetics of adsorption-induced conformational changes

Stopped-flow fluorescence spectroscopy is used to investigate the kinetics of the adsorption process. To my knowledge, this is the first demonstration of protein adsorption on solid spheres studied with the stopped-flow technique. The small size of the nanospheres allows the use of this technique, without the risk of blocking the small mixing channels in a stopped-flow apparatus. A typical example of a stopped-flow trace is shown in Fig. 2-4. The experiments are performed with different BLA/nanosphere ratios ranging from about 1100 to 160 BLA molecules for each nanosphere. This ratio covers the steep part of the adsorption isotherm as described before (Fig. 2-1). Below a ratio of 500, all BLA molecules are adsorbed on the polystyrene nanospheres and no free BLA is left in solution.

The stopped-flow traces are fitted to a double exponential equation, except for the measurement at the lowest BLA concentration where one exponential term suffices (Table 2-1). The rate constants observed are between 38 and 74 s⁻¹ for the fast phase (k_1) and between 6 and 10 s⁻¹ for the slower phase (k_2). Only k_1 depends on the BLA concentration. The major phase is the fastest phase and accounts for 30 to 35% of the total fluorescence change. About 50% to 70% of the fluorescence intensity increase occurs in the dead time of 15 ms (f_d in Table 2-1). However, the change of amplitude within the dead time is roughly as expected based on k_1 and currently does not suggest the presence of another faster phase.

Manual mixing experiments reveal a third phase that is responsible for 10% of fluorescence intensity increase, which occurs between 1 and 30 minutes after mixing BLA and the nanospheres (Fig. 2-5A). The increase in fluorescence intensity is accompanied by a shift in the fluorescence maximum from 326 to 336 nm within the first 15 seconds (Fig. 2-5A), the minimum time needed to obtain an emission wavelength scan. The gradual increase that occurs after the initial fluorescence burst depends on the emission wavelength (Fig. 2-6). At low emission wavelength (320 nm),

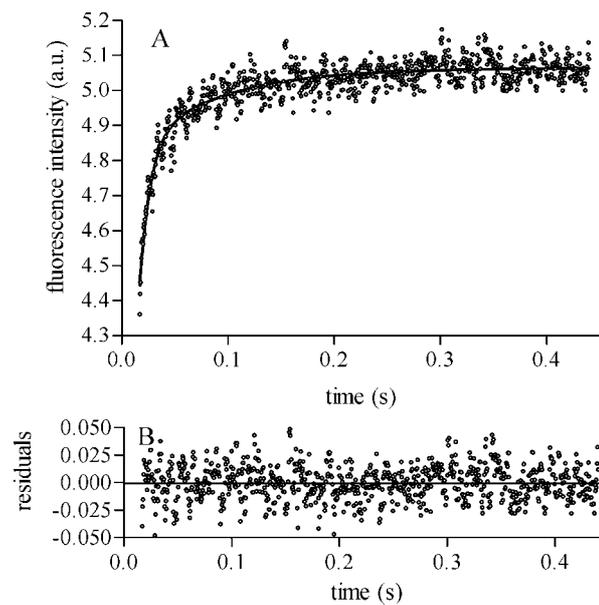


Fig. 2-4. Stopped-flow fluorescence trace (*circles*) of BLA adsorption on polystyrene nanospheres in a 10 mM Tris/HCl buffer with 1 mM CaCl_2 at pH 7.5 and at 20 °C (A). The BLA concentration was 4.0 μM and the nanosphere concentration was 5.0 nM. The fluorescence trace was fitted (*line*) with a double exponential function. Rate constants resulting from the fit are: $k_1 = 74 \text{ s}^{-1}$ and $k_2 = 7.5 \text{ s}^{-1}$. Residuals of the fit are shown in the lower panel (B).

the fluorescence intensity is constant between 12 seconds and 75 minutes after mixing BLA and the nanospheres. However, at higher emission wavelengths (340, 360 and 380 nm) the gradual increase in fluorescence intensity becomes more pronounced, which indicates the involvement of Trp residues in an exposed environment. The gradual increase in fluorescence intensity suggests slow structural rearrangements of adsorbed BLA molecules. The data at different emission wavelengths, representing fluorescence changes between 12 seconds and 75 minutes, were fitted separately to a single exponential function (Table 2-2). The rate constants for this phase are about $1 \cdot 10^{-3} \text{ s}^{-1}$. The near-UV CD trace of the manual mixing experiments shows that the change in conformation occurs within 10 seconds after the addition of the nanospheres (Fig. 2-5B). The ellipticity at 275 nm increases from the native value of $-350 \text{ deg cm}^2 \text{ dmol}^{-1}$ to the steady state value of $-150 \text{ deg cm}^2 \text{ dmol}^{-1}$ within 10 seconds, which is the time necessary to open the sample chamber, add the nanospheres, mix, and close the sample chamber again. Because of the low sensitivity of the CD method, it is not possible to track small changes in the signal that could occur after the initial increase of the signal.

In summary, the changes in fluorescence emission intensity induced by adsorption of BLA on the polystyrene nanospheres show the existence of three phases. The first phase has a rate constant of 38 to 74 s^{-1} depending on the BLA concentration. This phase contributes 22 to 37% to the total fluorescence emission intensity increase in a stopped-

Table 2-1. Fit results of the data obtained from stopped-flow fluorescence experiments of BLA adsorption on polystyrene nanospheres. The kinetic adsorption experiments were performed in a 10 mM Tris/HCl buffer of pH 7.5 with 1 mM CaCl₂ at 20 °C. The excitation wavelength was 300 nm. The dead time was 15 ms; n is the molar ratio BLA/nanospheres; p₀ is expressed as the total fluorescence amplitude (arbitrary units); f₁, f₂ and f_d are the amplitudes of the correspondent phases, expressed as fraction of the total amplitude; f_d is the amplitude in the dead time^a

| BLA (μM) | nanosphere (nM) | n | p ₀ | f ₁ ^b | k ₁ (s ⁻¹) ^b | f ₂ ^b | k ₂ (s ⁻¹) ^b | f _d ^b |
|----------|-----------------|------|----------------|-----------------------------|--|-----------------------------|--|-----------------------------|
| 0.80 | 4.97 | 161 | 0.87 | 0.53 | 38 | - | - | 0.47 |
| 1.34 | 4.97 | 270 | 1.46 | 0.36 | 47 | 0.17 | 7.2 | 0.47 |
| 1.87 | 4.97 | 376 | 1.76 | 0.37 | 48 | 0.11 | 6.6 | 0.52 |
| 2.27 | 4.97 | 457 | 1.81 | 0.36 | 51 | 0.09 | 6.3 | 0.55 |
| 2.67 | 4.97 | 537 | 2.10 | 0.31 | 68 | 0.11 | 8.5 | 0.58 |
| 3.07 | 4.97 | 618 | 1.99 | 0.30 | 74 | 0.10 | 10.1 | 0.60 |
| 4.00 | 4.97 | 805 | 2.43 | 0.22 | 74 | 0.07 | 7.5 | 0.71 |
| 2.63 | 2.33 | 1129 | 0.85 | 0.37 | 55 | 0.12 | 6.3 | 0.51 |
| 2.63 | 3.33 | 790 | 1.17 | 0.37 | 61 | 0.11 | 8.2 | 0.52 |
| 2.63 | 4.67 | 563 | 1.69 | 0.33 | 55 | 0.09 | 7.5 | 0.58 |
| 2.63 | 5.67 | 464 | 1.75 | 0.35 | 51 | 0.09 | 6.3 | 0.56 |
| 2.63 | 6.67 | 394 | 2.19 | 0.30 | 67 | 0.12 | 8.9 | 0.58 |
| 2.63 | 7.67 | 343 | 2.25 | 0.30 | 58 | 0.07 | 7.0 | 0.63 |
| 2.63 | 10.00 | 263 | 3.04 | 0.25 | 67 | 0.08 | 9.7 | 0.67 |

^a Stopped-flow fluorescence data are fitted according to $I(t) = p_0 + p_1 e^{(-tk_1)} + p_2 e^{(-tk_2)}$.

^b The standard deviations are ± 0.03 for f values, ± 3 s⁻¹ for k₁ and ± 1 s⁻¹ for k₂. The standard deviations are based on the results of three separate fits on the time-dependent fluorescence trace. Each trace is the average of ten individual measurements.

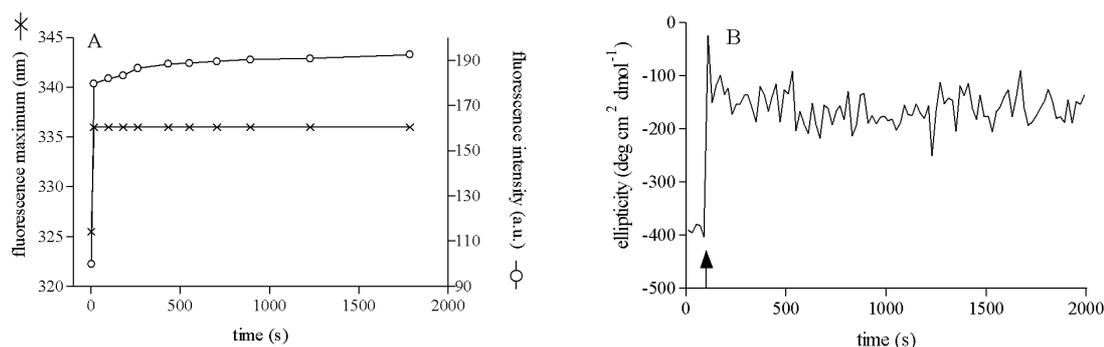


Fig. 2-5. Manual mixing experiments in which BLA is adsorbed on polystyrene nanospheres in a 10 mM Tris/HCl buffer with 1 mM CaCl₂ at pH 7.5 and at 20 °C. (A) Normalized fluorescence intensity at 330 nm (circles, right axis) and fluorescence spectral maximum (crosses, left axis) after addition of polystyrene nanospheres (1 nM) to a BLA (0.5 μM) solution at t = 0 s. (B) Molar ellipticity at 275 nm of BLA (4.5 μM) adsorption on polystyrene nanospheres (10 nM). The arrow indicates the moment of addition of the nanospheres to the BLA solution. The initial peak after the addition of the nanospheres is caused by opening of the sample chamber during 10 seconds, and should not be considered as relevant data.

flow trace and is also responsible for the 50 to 70% increase in fluorescence emission intensity during the dead time of the experiment. The phase is associated with the major unfolding of BLA in its native state to an intermediate folding state which is adsorbed on the polystyrene nanospheres and which has molten globule-like characteristics. Thus, a state with preserved secondary structure and disrupted tertiary structure already exists shortly after adsorption of BLA on the nanospheres. The second and third, slower phases are responsible for about 10% of the fluorescence signal change and have rate constants of between 6 to 10 s⁻¹ (k_2) and of about 0.001 s⁻¹ (k_3), respectively.

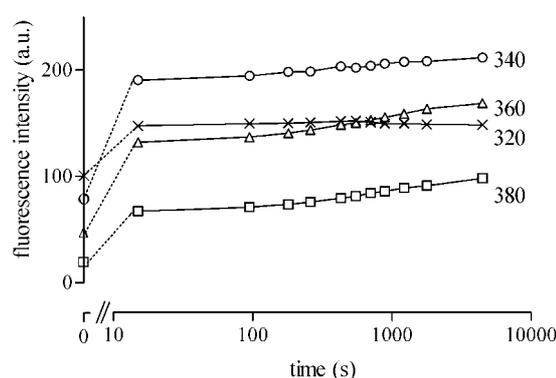


Fig. 2-6. Normalized fluorescence intensity at different emission wavelengths (320, 340, 360 and 380 nm) during the adsorption of BLA (0.5 μ M) on polystyrene nanospheres (1 nM). Experiments were done in a 10 mM Tris/HCl buffer with 1 mM CaCl₂ at pH 7.5 and at 20 °C. The manual mixing method resulted in a dead time of approximately 10 seconds. Polystyrene nanospheres are added to a BLA solution at $t = 0$ s. Excitation is at 300 nm. The fluorescence intensity of BLA in solution at an emission wavelength of 320 nm is normalized to 100. The other data points have been normalized correspondingly.

Table 2-2. Fit results of the data obtained from manual mixing fluorescence experiments of BLA adsorption on polystyrene nanospheres. The adsorption experiments were performed in a 10 mM Tris/HCl buffer of pH 7.5 with 1 mM CaCl₂ at 20 °C. The excitation wavelength was 300 nm. The dead time was about 10 seconds; λ_{em} is the emission wavelength; p_0 is expressed as the total fluorescence amplitude (arbitrary units), the amplitude f_3 is expressed as fraction of the total amplitude, k_3 is a rate constant for unfolding.^a

| λ_{em} (nm) | p_0 | f_3^b | k_3 (s ⁻¹) ^b |
|---------------------|-------|---------|---------------------------------------|
| 320 ^c | - | - | - |
| 340 | 244 | 0.15 | 0.0016 |
| 360 | 408 | 0.28 | 0.0011 |
| 380 | 654 | 0.36 | 0.0010 |

^a The time-dependent fluorescence data are fitted according to the equation $I(t) = p_0 + p_3 e^{-tk_3}$

^b Standard deviations are ± 0.03 for f values and ± 0.0002 s⁻¹ for k_3 based on two separate experiments

^c No fit was made because the data yielded a straight, horizontal line

2.3.5 Diffusion and association do not affect the observed stopped-flow fluorescence traces

It has been recognised that three processes must play an important role during the complex kinetics of protein adsorption and subsequent unfolding (Andrade, et al., 1986). First, the BLA molecules have to find their way to the surface of the polystyrene nanospheres. This process is governed by the kinetics of mass transport: both convective transport and diffusion play an important role. Since mixing in the stopped-flow apparatus is virtually ideal, convective transport will not be considered. Secondly, the protein molecules that are in the vicinity of the interface should adsorb on the interface. The rate of this association process is governed by several factors, including the sticking probability and the coverage of the adsorbent surface. The third process involves conformational changes of the adsorbing protein, which is the subject of this paper. The previously discussed stopped-flow fluorescence traces that led to the conclusions about the conformational changes of adsorbing BLA molecules might be interwoven with the diffusion controlled process and/or with the association controlled process. These processes would become visible in the stopped-flow fluorescence traces when the conformational change, which leads to increased fluorescence intensity, would be much faster than the rate of diffusion or association. The possibility of diffusion-limited processes affecting the stopped-flow fluorescence traces in these experiments should be addressed, since especially the size of the polystyrene nanospheres is large compared to the size of a BLA molecule, and since their concentration is much lower than for example the GdnHCl concentration in a common stopped-flow unfolding experiment.

In case diffusion is a limiting factor in the kinetic process, its contribution can be estimated. The maximum rate of adsorption of a protein on a sphere for a diffusion-limited process can be calculated by using Equation 2-1 (Atkins, 1990). The equation is derived from a calculation of the average flow of protein molecules to a sphere.

$$k_{\text{diff}} = 4\pi N_A R_s D_p \quad (\text{Eq. 2-1})$$

In this equation k_{diff} is the diffusion-limited association rate constant ($\text{M}^{-1}\text{s}^{-1}$), N_A is Avogadro's number (mol^{-1}), R_s is the radius of the sphere (m) and D_p is the diffusion coefficient of the protein (m^2/s). For the adsorption of BLA on polystyrene nanospheres (radius 24 nm, diffusion coefficient $1.06 \times 10^{-10} \text{ m}^2/\text{s}$ as determined by dynamic light scattering) a diffusion-limited association rate constant of $1.9 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ is calculated. For the protein concentrations used, diffusion-limited association rates between $15,000 \text{ s}^{-1}$ and $75,000 \text{ s}^{-1}$ are calculated. These rates are much higher than the ones found in the

analysis of the stopped-flow fluorescence traces (Table 2-1). Consequently, diffusion does not affect the stopped-flow data presented here.

Equation 2-1 is based on the assumption that the concentration of protein in solution is constant, but this is not the case in these experiments. On the contrary, the protein concentration decreases and in some cases even drops to zero in the course of the adsorption process. As a result, the rate of diffusion would also decrease when more and more protein molecules are adsorbed and thus less BLA is present in solution. However, calculations show that even when the protein concentration has dropped to 1% of its original value, the diffusion-limited rate is still much higher than the fastest process observed here.

As indicated, the rate of association of the proteins to the nanospheres is another factor that could affect the observed unfolding rates. Since the surface of the polystyrene nanospheres is increasingly covered by BLA molecules during adsorption, and since the protein concentration decreases during adsorption, it is expected that the association rate decreases in time. In the experiments described here, a protein-to-nanosphere ratio is used such that virtually all molecules adsorb and form a monolayer on the polystyrene surface. This means that the protein concentration in solution drops to almost zero and coverage of the surface increases to almost its maximum during adsorption. Unfortunately, whether an association-limited phase affects the interpretation of the presented stopped-flow fluorescence data cannot easily be predicted.

To confirm that the measured fluorescence traces as shown in the previous section are not affected by diffusion and/or association processes, fluorescence anisotropy has been measured during the adsorption process. The idea is that adsorption of BLA on the relatively large nanospheres will drastically decrease the rotational correlation time of BLA and thus lead to an increase of its fluorescence anisotropy. A calculation based on the Perrin equation shows the relation between the fluorescence anisotropy (r) and the rotational mobility of the fluorophore (Equation 2-2).

$$\frac{r_0}{r} = 1 + \frac{\tau}{\phi} \quad \text{Eq. 2-2}$$

In this equation r_0 is the fundamental anisotropy of the fluorophore, r is the measured anisotropy, τ is the fluorescence lifetime (s) and ϕ is the rotational correlation time (s). The fluorescence lifetime of BLA is about 2.6 ns and the fundamental anisotropy of tryptophan is about 0.30 at an excitation wavelength of 300 nm (Lakowicz, 1999). The rotational correlation time for both BLA and nanospheres can be estimated based on their radius and on the assumption of both being spherical particles. This yields ϕ values

of 5 ns for BLA and of about 1 μ s for the nanospheres. Using equation 2-2, the anisotropy of free and adsorbed BLA is predicted to be 0.20 and 0.30, respectively. The intrinsic fluorescence anisotropy is measured for the adsorption of BLA on polystyrene nanospheres, both in manual mixing experiments and in stopped-flow experiments. The results show that the anisotropy of free BLA increases from the value of 0.18 for BLA in solution to a steady state value of 0.29 after addition of the nanospheres within the dead time of the manual mixing experiment, which is about 1 minute (Fig. 2-7A). In a stopped-flow experiment, the anisotropy of free BLA mixed with buffer is 0.09 and remains constant, which is not surprising since no change in the rotational correlation time of the free BLA is anticipated (Fig. 2-7B). After mixing BLA with the nanospheres the anisotropy is 0.22 and it remains constant during the 2 seconds the stopped-flow experiment lasts. Whereas the anisotropy measured by the manual mixing experiments agree very well with the predicted values, those extracted from the stopped-flow experiments do not. However, the increase in anisotropy upon BLA adsorption is for both experiments nearly equal (i.e. about 0.12). Slight deviations in the wavelength and the bandpass of the excitation light between the manual mixing experiment and the stopped-flow experiment most likely cause the observed differences in the anisotropy values. The anisotropy of proteins containing tryptophan residues shows large differences upon altering the excitation wavelength between 295 and 300 nm (Lakowicz, 1999).

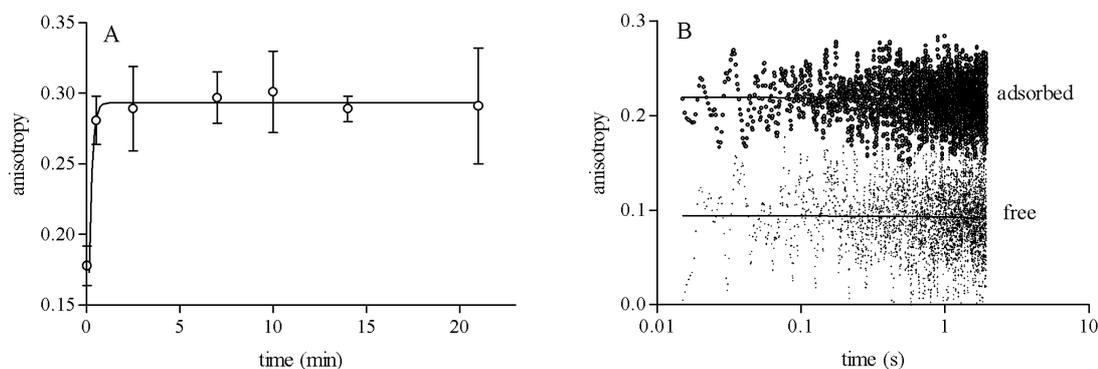


Fig. 2-7. Time-dependent fluorescence anisotropy during adsorption of BLA on polystyrene nanospheres determined by a manual mixing experiment (A) and by a stopped-flow experiment (B). The experiments were performed in a 10 mM Tris/HCl buffer with 1 mM CaCl_2 at pH 7.5 and at 20 °C. The BLA concentration was 0.5 μ M and the nanosphere concentration was 1 nM in the manual mixing experiment, whereas the BLA concentration was 4.5 μ M and the nanosphere concentration was 10 nM in the stopped-flow experiment. The polystyrene nanospheres were added to the BLA solution at $t=0$. The stopped-flow traces were fitted with a straight line, which resulted in anisotropy values of 0.094 for BLA free in solution and of 0.219 for BLA adsorbed on polystyrene nanospheres.

Despite the differences in the absolute values of anisotropy obtained from manual mixing and stopped-flow experiments, the stopped-flow experiment clearly indicates an increased rotational correlation time upon adsorption of BLA on the nanospheres. The increase occurs within the dead time of the experiment, indicating that BLA is adsorbed within 15 ms. Consequently, the stopped-flow fluorescence traces are not affected by diffusion or association processes, but instead can be completely attributed to the unfolding of BLA adsorbed on the polystyrene nanospheres.

Since removal of calcium from BLA facilitates the formation of the molten globule state, it was tested whether adsorbed BLA still contains calcium. Besides adsorbing proteins, it was found that the nanospheres also adsorb calcium ions. Hence, it is not possible to detect free calcium ions in solution that are released from adsorbed BLA molecules. The K_d for the interaction of calcium ions with the polystyrene nanospheres was determined (Fig. 2-8). A Langmuir fit of these data results in a K_d of $16 \pm 5 \mu\text{M}$, indicating that the interaction between calcium ions and the nanospheres is quite strong. The K_d for the interaction of calcium with the molten globule state of BLA is approximately 1 mM (Kuwajima, et al., 1989). Consequently, calcium ions cannot be present in the calcium binding site of the adsorbed BLA molecules, but are instead adsorbed on the surface of the nanospheres.

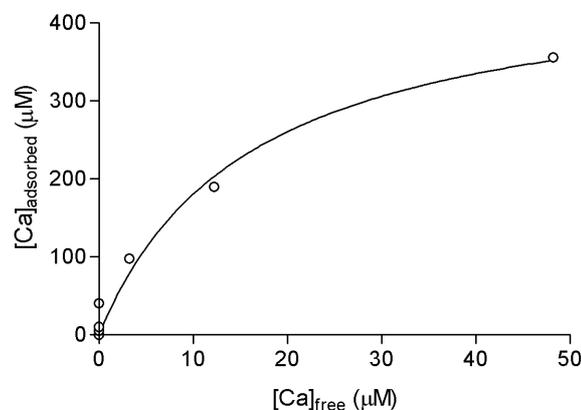


Fig. 2-8. Adsorption isotherm of calcium on polystyrene nanospheres in a 10 mM Tris/HCl buffer at pH 7.5 after 20 hours of adsorption (data (circles), fit (line)). The concentration of the nanospheres is 80 nM. Each data point is the average of three measurements. The filtrate (Amicon 50 kD ultrafiltration) is analysed for calcium content by ICP-OES. The data are fitted with a Langmuir equation: $[\text{Ca}]_{\text{adsorbed}} = (B * [\text{Ca}]_{\text{free}}) / (K_d + [\text{Ca}]_{\text{free}})$, resulting in a K_d of $16 \pm 5 \mu\text{M}$, a B value of $4.7 * 10^2 \pm 0.6 * 10^2 \mu\text{M}$, and a R^2 value of 0.98. In the latter equation, B is the plateau value of adsorption, and K_d is the dissociation constant.

2.4 DISCUSSION

In this study, it is shown that the kinetics of adsorption-induced conformational changes can be determined with stopped-flow fluorescence spectroscopy. The main

unfolding event is remarkably fast and results in a partially unfolded state. The spectroscopic observations made here for the adsorbed state of BLA, i.e. tertiary structure loss but persistent secondary structure, are known to be characteristic for a molten globule state, which is an intermediate in the folding pathway of BLA in solution (Kuwajima, 1996; Permyakov and Berliner, 2000). A molten globule state has increased exposure of hydrophobic groups, which can be measured by hydrophobic fluorescent probes like ANS (Semisotnov, et al., 1991). In addition, it has been shown that adsorption of BLA on a hydrophobic surface like polystyrene occurs via hydrophobic interactions (Lindahl and Vogel, 1984; Noppe, et al., 1999; Norde and Anusiem, 1992a). The observation that adsorbed BLA has a molten globule conformation with exposed hydrophobic groups supports the latter. Furthermore, it is shown here that calcium cannot be present in adsorbed BLA, which further supports the presence of an adsorbed molten globule state of BLA.

2.4.1 *Orientation of BLA molecules on the nanosphere surface*

Despite the importance of hydrophobic interactions for BLA adsorption, it is not likely that all four tryptophans of adsorbed BLA are in close contact with the hydrophobic polystyrene surface (dielectric constant = 2.6). This is supported by the observed red shift of the fluorescence emission maximum, indicating that one or more Trp residues are more exposed to the solvent (Fig. 2-2). A number of hydrophobic residues must be in close contact with the hydrophobic polystyrene surface for binding through hydrophobic interactions to occur. The preference of these residues for a position close to the hydrophobic polystyrene surface results in a specific conformation of the BLA molecule on the surface, which affects the environment of the tryptophan residues. Investigation of the crystal structure of BLA can give us a clue about the preferred orientation of BLA on a hydrophobic interface. At pH 7.5 both BLA and the nanosphere surface have a net negative charge. The negative charge on the surface of a BLA molecule is concentrated in a patch near the calcium-binding site. This patch includes aspartic acid residues 14, 63, 64, 78, 82, 83, 84, 87 and glutamic acid residues 1, 7 and 11. The most likely orientation of a BLA molecule approaching a negatively charged interface would thus be with the negative patch facing towards the solution, pointing away from the interface. Note that this orientation would not hinder calcium release of BLA in the adsorbed state. In addition, a possible candidate for orientation of BLA by hydrophobic interactions is the hydrophobic patch formed by the 'flexible loop' (residues 105-111) and the adjacent aromatic cluster I (residues 31, 32, 117, 118) (Pike, et al., 1996). Both hydrophobic regions are thought to be involved in binding of BLA to galactosyl transferase, resulting in the enzyme complex lactose synthase. The

hydrophobic patch is on the opposite side of the calcium-binding site, thereby enabling orientation by both electrostatic and hydrophobic interactions.

2.4.2 Scheme for the adsorption of BLA on polystyrene nanospheres

In Fig. 2-9 a scheme is proposed for the adsorption and unfolding processes that play a role during adsorption of BLA on the polystyrene nanospheres. In solution, the native state of BLA (N_s) is in equilibrium with an intermediate state (I_s), which is generally known to be a molten globule state. Two routes are depicted that lead from the start situation, native BLA (N_s), to the end situation of adsorbed and partially unfolded BLA (D_a). The first route involves the adsorbed native state N_a , while the second route includes the I_s state. An argument in favour of the second route is the hydrophobic nature of the intermediate state of α -lactalbumin in solution, which would more easily adsorb on a hydrophobic surface than the native state does. However, the population of the intermediate state is very low in solution so the chance that the intermediate state comes in proximity of a nanosphere is very small. The rate of GdnHCl induced unfolding of native BLA to the intermediate state I_s is about 0.001 s^{-1} when extrapolated to zero M GdnHCl (Kuwajima, et al., 1989). If the adsorption and subsequent unfolding route would proceed via I_s , the observed rate of unfolding would be affected by this step. Since the rate constant found here is four orders of magnitude larger, the latter route can be excluded as indicated by the shaded areas in the scheme.

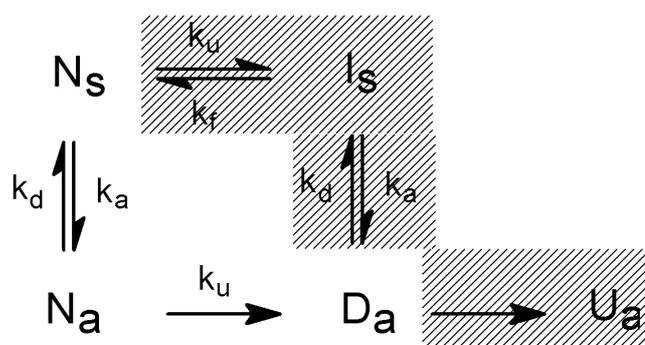


Fig. 2-9. Schematic model for adsorption-induced unfolding of bovine α -lactalbumin on a polystyrene interface. N is the native state, I is the intermediate state (molten globule state), U is the completely unfolded state and D is a denatured state which strongly resembles the molten globule state of BLA in solution. Subscript 's' indicates the species in solution and subscript 'a' indicates adsorbed species. The folding and unfolding rate constants k_f and k_u are shown, as well as the adsorption rate constant k_a and desorption rate constant k_d . The shaded areas indicate parts of the scheme that do not play a significant role in the adsorption of BLA, as shown by the results presented in this paper (see text for further details).

The most likely route for BLA adsorption involves the adsorbed native state N_a . After adsorption, the native state is partially unfolded to a denatured state D_a . The denatured state is characterized by conserved secondary structure, lost tertiary structure, and loss of the calcium ion. The adsorbed denatured state has the typical characteristics

of the molten globule state of BLA (I_s), which is an intermediate during the folding of the protein. The adsorption-induced partial unfolding of N_a occurs rapidly as 50 to 70% of the fluorescence intensity increase already occurs in the 15 ms dead time of the experiment and a further 30 to 35% increase has a rate constant of 38 to 74 s^{-1} . It is interesting to compare the adsorption-induced unfolding rates with the rate of BLA unfolding induced by common denaturants like guanidine hydrochloride (GdnHCl). The rate of GdnHCl induced unfolding of native BLA at 5.6 M GdnHCl is about 4 s^{-1} (Kuwajima, et al., 1989). The observation made here is similar: when BLA is unfolded in 5.2 M GdnHCl the fluorescence unfolding trace could be fitted to a single exponential function with a rate constant of $2.4 \pm 0.2 s^{-1}$. The unfolding of BLA on polystyrene nanospheres has a remarkably fast rate of 50 s^{-1} or higher as shown by the experiments in this chapter. The adsorbed and partially denatured state has stable interactions with the polystyrene interface and desorption hardly occurs. The polystyrene/water interface thereby acts like a local sink, or more probably like a series of local sinks, that stabilise folding intermediates located somewhere in the folding energy landscape between the native state and the array of completely unfolded species of BLA.

It is unlikely that a single population of denatured species with identical conformational properties exists in the adsorbed state. There are many factors involved that make the adsorption process different for individual molecules. First of all the homogeneity of the polystyrene/water interface needs not be perfect, one can think of physical irregularities and unequal distribution of charged surface groups. The first arriving BLA molecules will predominantly occupy sites that lead to the lowest free energy of the adsorbed molecules. Secondly, when the interface becomes more crowded other factors become important, for instance protein-protein interactions. Whereas the major unfolding phase with rate k_1 is most probably related to the transition of the native state to the partly denatured state, it is much harder to assign the slower phases k_2 and k_3 . Most probably, the latter phases are caused by subsequent local unfolding processes of the partly denatured, adsorbed BLA molecules, which do not affect the secondary structure and influence only the Trp environments. It is also possible that BLA molecules that adsorb when the interface is almost covered (but still in the dead time of the experiment) unfold much slower because of steric hindrance or protein-protein interactions. However, as k_2 does not depend on the BLA concentration, the latter possibility seems not very likely.

As indicated in the proposed scheme, it is further assumed that the desorption rate of the partly denatured, adsorbed BLA (D_a) is negligible. Upon adsorption of a protein molecule there will most likely be a number of interacting sites between the protein molecule and the interface (Andrade, et al., 1986). As is known from protein

chromatography, a too high site density on a chromatographic support can lead to irreversible adsorption of the protein. The experiments show that desorption of BLA molecules does not occur upon washing with buffer, even after 24 hours. Completely unfolded adsorbed species are omitted from the scheme for adsorption of BLA since the fluorescence maximum indicates that the adsorbed BLA molecules are not completely unfolded.

2.4.3 *Concluding remarks*

Unlike heat-, pressure- or denaturant-induced protein unfolding, adsorption-induced unfolding of proteins has received little attention. An important cause for the latter is the impossibility to acquire classical equilibrium unfolding curves like those routinely obtained for denaturant-induced protein unfolding. In fact, adsorption under the conditions described in this chapter is a nearly irreversible thermodynamic process. Consequently, a thermodynamic analysis of the data similar to the one applied to classical equilibrium unfolding data is impossible. As opposed to protein unfolding by high concentrations of a conventional denaturant, adsorption-induced protein unfolding, as shown here for BLA, can already be achieved by the addition of only 5 nM of polystyrene nanospheres, which corresponds to about 2.5 μM interaction sites. It is thus a major challenge to obtain detailed information on the molecular level about adsorption-induced protein unfolding. The importance of the phenomenon of protein adsorption in everyday life further warrants the investigation of adsorption-induced protein unfolding.

Here it is shown by tryptophan fluorescence spectroscopy, far-UV and near-UV CD spectroscopy and by the observation of the release of calcium that adsorption of bovine α -lactalbumin on polystyrene nanospheres leads to a denatured state of the BLA molecule which has the characteristics of a molten globule. The time-dependent conformational changes observed upon adsorption of BLA are not affected by diffusion or association processes. The rate of partial unfolding of adsorbed BLA molecules is remarkably fast (38 to 74 s^{-1}) and exceeds significantly the rate of BLA unfolding induced by the addition of a high concentration of denaturant, i.e. 5.4 M GdnHCl.

The results presented here contribute to the understanding of protein unfolding that is induced by the interaction of a protein with an interface. To obtain further information about the conformational characteristics of adsorbed BLA, hydrogen-deuterium exchange experiments on adsorbed BLA are envisioned. The exchange of labile hydrogen atoms of adsorbed BLA will then be monitored by NMR in the desorbed state of the protein.

3 Refolding of adsorbed bovine α -lactalbumin during surfactant-induced displacement from a hydrophobic interface

Little is known about the changes in protein conformation that occur after displacement of a protein from an interface. Here, results are presented that give insight in the conformation of bovine α -lactalbumin (BLA) molecules that are displaced from a hydrophobic polystyrene interface. After the BLA molecules are adsorbed on polystyrene nanospheres, they are displaced from these nanospheres using two surfactants: Tween 20 and CHAPS. The properties of displaced BLA depend on the concentration of the surfactant used to displace the protein and on the incubation time during displacement, as can be concluded from intrinsic fluorescence spectroscopy, circular dichroism spectroscopy, and non-denaturing gel electrophoresis. CHAPS is more effective in displacing adsorbed BLA than Tween 20. The largest amount of displaced BLA (90% recovery) is obtained at a CHAPS concentration of 2 mM or higher. At a surfactant concentration of 1 or 2 mM, displaced BLA contains calcium and has native spectroscopic properties, indicating that BLA, which has a molten globule-like conformation in the adsorbed state, refolds to its native state upon displacement from the surface. However, non-native properties of displaced BLA are observed at a low surfactant concentration (0.3 mM) after prolonged incubation. Under these conditions, the ensemble of displaced BLA molecules contains calcium, has native-like secondary structure, non-native tertiary structure, and contains a population of molecules that has a higher electrophoretic mobility on non-denaturing gels compared to native BLA. Intramolecular disulfide shuffling can cause the observed conformational changes. The disulfide shuffling is initiated by a few reactive groups on the surface of the nanospheres. It occurs during the homomolecular exchange of proteins at a surfactant concentration of 0.3 mM and is time-dependent. Both Tween 20 and CHAPS are good candidates for the removal of proteins from interfaces, as long as the incubation time is short and the surfactant concentration is above a certain threshold. The displacement procedure presented here is essential for the future study of the atomic details of the conformation of proteins adsorbed on interfaces using NMR spectroscopy in combination with H/D exchange measurements.

3.1 Introduction

Many proteins present in nature or in man-made systems are located at an interface, for example a membrane protein at the membrane interface or a serum protein adsorbed on a medical implant. It is generally accepted that adsorption on an interface affects the conformation of proteins. The conformation of proteins adsorbed on interfaces is the subject of studies in bio-science as well as in other fields, like food science, medical science and chromatography (Andrade, et al., 1986; Cawthorn, et al., 1996; Killian and von Heijne, 2000; Norde, 1999). Proteins adsorbed on an interface have altered conformations, ranging from an increase in secondary structure to the loss of nearly all secondary structure (Maste, et al., 1996; Smith, et al., 1992). Recently, it has been suggested that adsorption of amyloid proteins on an interface induces conformational changes, resulting in amyloid fibril formation (Kowalewski, et al., 1999; Schladitz, et al., 1999). The non-native conformation of adsorbed and possibly of desorbed or displaced proteins can affect their properties, and consequently, their function.

In this study, information about the conformation of bovine α -lactalbumin (BLA) after its displacement from a hydrophobic interface by a surfactant is obtained. BLA is chosen for two reasons. Firstly, its structure, stability, and folding behaviour have been thoroughly studied. Secondly, the adsorption behaviour of BLA on a variety of interfaces is known. In chapter 2 it was shown that BLA which is adsorbed on a hydrophobic interface has a molten globule-like (MG) conformation, with conserved secondary structure and lost tertiary structure (Engel, et al., 2002). It is however not clear whether the adsorption-induced conformational changes of BLA are permanent or whether they are reversed upon displacement of the protein. In addition, a procedure for controlled protein displacement from an interface is essential for the future study of the atomic details of adsorption-induced protein unfolding by NMR spectroscopy and hydrogen exchange methods.

Here, two surfactants are used to displace BLA from a hydrophobic surface: Tween 20 (polyoxyethylenesorbitan monolaurate) and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate). Tween 20 is able to displace proteins from liquid (Courthaudon, et al., 1991; Mackie, et al., 1999; Wilde and Clark, 1993) and solid interfaces (Elwing, et al., 1989; Feng, et al., 1995). No data about protein displacement with CHAPS are available. However, CHAPS is well known as a solubilizer of membrane proteins (Hjelmeland, 1990). Both surfactants are known to preserve the native structure of proteins. Although some information exists on the mechanisms of protein displacement (Clark, et al., 1991; Mackie, et al., 1999; Wilde, et al., 1993), little is known about the conformation of displaced proteins.

Both ‘displacement’ and ‘desorption’ are used as expressions to indicate the detachment of molecules from an interface. Here, displacement is defined as the process in which molecules – surfactants, small proteins, or even the same protein molecules as the adsorbed ones – remove adsorbed protein molecules by competing for adsorption on the interface. The process in which proteins of the same kind displace each other is also known as homomolecular exchange (Norde, et al., 2000). In contrast, desorption is defined as the process in which protein molecules detach from an interface under conditions similar to the ones in which adsorption took place. BLA does not spontaneously desorb from polystyrene nanospheres, even after washing with buffer. Here, only BLA displacement is thus considered.

In this study, BLA is first adsorbed on hydrophobic polystyrene nanospheres and subsequently displaced from the nanospheres by adding a surfactant. The properties of displaced BLA are studied using intrinsic fluorescence spectroscopy, circular dichroism spectroscopy (CD), and non-denaturing gel electrophoresis. Insight is obtained in the conformational changes that occur upon BLA displacement and in the role the surfactant plays during BLA displacement.

3.2 Materials and methods

3.2.1 Materials

Bovine α -lactalbumin (Sigma L-5385) was used without further purification. CHAPS (Sigma C5070) and Tween 20 (Merck) were used as surfactants. Polystyrene nanospheres, supplied as a colloidal suspension in water, were obtained from Polymer Laboratories (Heerlen, The Netherlands). All other chemicals were of analytical grade. Nanosphere suspensions were diluted with buffer before use. All experiments were done in a 10 mM Tris/HCl buffer at pH 7.5 that contained 1 mM CaCl_2 , unless specified otherwise. Calcium determinations were done in a calcium-free 10 mM Tris/HCl buffer at pH 7.5. Nanopure water was used for the preparation of the buffers (Sybron Barnstead NANOpure II).

3.2.2 Determination of the amount of displaced BLA

BLA (30 μM) was incubated with polystyrene nanospheres (100 nM) in a volume of 2 – 10 ml at room temperature for 30 minutes. This time period suffices for complete BLA adsorption (Engel, et al., 2002). The protein was displaced through the addition of a surfactant, during different incubation times, ranging from 20 minutes to 16 hours. The displacement procedure was stopped by separating displaced BLA molecules from the polystyrene nanospheres using ultrafiltration filters with a 100 kD molecular weight cut off (Centricon YM 100, Millipore Corporation; Vivaspin 4 Concentrator,

Vivascience). Initially, ultrafiltration was done using Centricon filters, but later on Vivaspin filters were used because they have a shorter filtration time, which results in shorter surfactant incubation times. The concentration of BLA was measured spectrophotometrically using a molar extinction coefficient of $28,540 \text{ M}^{-1}\text{cm}^{-1}$ for BLA at 280 nm. Reference samples, containing surfactant and nanospheres, were used as a blank. Protein adsorption on the filter was checked and found to be negligible. The filter retained more than 99.8% of the polystyrene nanospheres. The remaining 0.2% of nanospheres present in the samples did not affect the fluorescence, CD and non-denaturing gel electrophoresis experiments. The amount of recovered BLA is defined as the amount of BLA in the filtrate after ultrafiltration.

3.2.3 Calcium quantification

Samples containing either nanospheres, BLA, surfactant, calciumchloride, or combinations of these were ultrafiltered (Amicon, YM50 membrane, Millipore Corp.) and the filtrate was subsequently analyzed for calcium content by Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES). The BLA concentration in the filtrate was determined spectrophotometrically, as described above.

3.2.4 Fluorescence spectroscopy

Fluorescence spectra were measured in a quartz cuvette (10 * 4 mm) on a Cary Eclipse fluorimeter (Varian). The temperature of the cuvette holder was maintained at 20 °C using a Peltier element during all experiments. The excitation wavelength was 300 nm, with excitation slits set at 2.5 nm and emission slits set at 5 nm. A fluorescence spectrum of a blank, which contained all components except protein, was subtracted from the fluorescence spectra of the samples. The total absorbance of the samples was kept below 0.1 at a wavelength of 300 nm to minimize the inner filter effect. Unfolded BLA was prepared by adding a solution of 8.0 M guanidine hydrochloride (GdnHCl) to native BLA resulting in a final GdnHCl concentration of 7.1 M. The MG-state of BLA was prepared by dissolving BLA in water, the pH of which was adjusted to 2 using concentrated HCl. Several samples of displaced BLA were unfolded by the addition of a solution of 8.0 M GdnHCl (resulting in a final GdnHCl concentration of 5.2 M) and subsequently refolded by dilution with buffer (resulting in a final GdnHCl concentration of 0.9 M). These refolded samples were concentrated on 3 kD Centricon ultrafiltration devices. Before fluorescence spectra were measured, 1 mM CaCl_2 was added to the samples of displaced BLA, resulting in a final CaCl_2 concentration between 1 and 2 mM.

3.2.5 Circular Dichroism spectroscopy

CD measurements were performed on a Jasco J-715 spectropolarimeter, equipped with a Peltier temperature control system set at 20 °C. Calibration was performed with a solution of ammonium d-10-camphorsulfonate in nanopure water of which the concentration (0.060% (w/v)) was checked spectrophotometrically. Typical protein concentrations used were 2 to 7 μM in a 0.1 cm quartz cuvette for far-UV CD and 5 to 22 μM in a 1 cm quartz cuvette for near-UV CD measurements. Eight scans were averaged for each sample. The response time was 1 second, the spectral band width was 1.0 nm. Before analysis of the spectra, a CD spectrum of a blank, which contained all components except BLA, was subtracted from the CD spectra of the samples. The spectra were smoothed using Jasco software. Before CD spectra were measured, 1 mM CaCl_2 was added to the samples of displaced BLA, resulting in a final CaCl_2 concentration between 1 and 2 mM.

3.2.6 Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed on a Mighty Small II system (Hoefer Scientific Instruments) with 10%T, 3%C gels, including a 4%T, 3%C stacking gel. Non-denaturing gels contained 1 M Tris adjusted to pH 8.5 with concentrated HCl. The non-denaturing electrophoresis buffer contained 5 mM Tris and 38 mM glycine, resulting in a pH of 8.7. SDS-PAGE was performed according to the procedure described by Schagger (Schagger and von Jagow, 1987). The gels were stained with Coomassie blue.

3.3 Results

3.3.1 The displacement of BLA

In this study, BLA molecules are first adsorbed on polystyrene nanospheres and subsequently displaced from these nanospheres using a surfactant. The two surfactants employed are Tween 20 and CHAPS. The efficiency of displacement is studied at different surfactant concentrations. The amount of BLA adsorbed is about 60% of the monolayer concentration, which corresponds to about 300 BLA molecules per nanosphere (Engel, et al., 2002). After displacement, intrinsic fluorescence and CD spectra are acquired to determine the conformation of displaced BLA.

Previous studies show that the conformation of adsorbed BLA is not native, but instead resembles that of an MG state (Engel, et al., 2002). It is unknown whether this conformational state lasts after BLA is removed from the nanosphere surface. It is expected however, that the conformation of displaced BLA will be native, since the driving force for adsorption-induced unfolding – the interaction of BLA with the

hydrophobic interface – is not present after displacement. As a result, the adsorption-induced MG-state should refold to the native state after displacement.

Both Tween 20 and CHAPS are able to displace adsorbed BLA from the surface of polystyrene nanospheres (Fig. 3-1). CHAPS is more effective in displacing adsorbed BLA than Tween 20. The largest amount of displaced BLA (i.e. 90% recovery) is obtained at a CHAPS concentration of 2 and 10 mM. Addition of Tween 20 results in a maximum BLA recovery of 50% at a Tween 20 concentration of 1 to 2 mM. BLA is not displaced below a surfactant concentration of 0.1 mM. Surprisingly, the displaced amount of BLA decreases at Tween 20 concentrations exceeding 5 mM. Additional experiments show that at these high Tween 20 concentrations, protein-surfactant complexes of BLA and Tween 20 molecules are formed, which are not able to pass the 100 kD filter used to separate the displaced protein from the polystyrene nanospheres (data not shown). As a result, not all displaced BLA molecules are recovered in the filtrate at Tween 20 concentrations exceeding 5 mM.

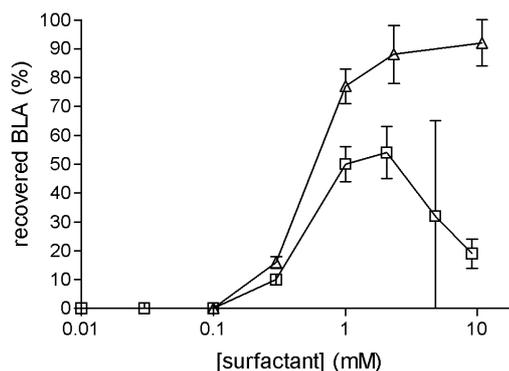


Fig. 3-1. Recovered amount of BLA molecules that are first adsorbed on polystyrene nanospheres and subsequently displaced using different concentrations of CHAPS (triangles) and Tween 20 (squares). The recovered amount of BLA is expressed as a percentage of the initial amount of native BLA molecules (30 μ M) that was added to the nanospheres (100 nM). The incubation time in the presence of the surfactant is 90 minutes. The error bars are the standard deviations calculated from 3 to 6 individual measurements. The experiment is performed at room temperature. The calcium concentration is 1 mM.

3.3.2 The conformation of displaced BLA

The conformational properties of displaced BLA, after separation from the nanospheres by ultrafiltration, are studied using intrinsic fluorescence spectroscopy, circular dichroism spectroscopy and non-denaturing gel electrophoresis. The fluorescence emission maxima and the corresponding fluorescence intensities at these emission maxima of BLA displaced by different amounts of Tween 20 and CHAPS are shown in Figs. 3-2 and 3-3.

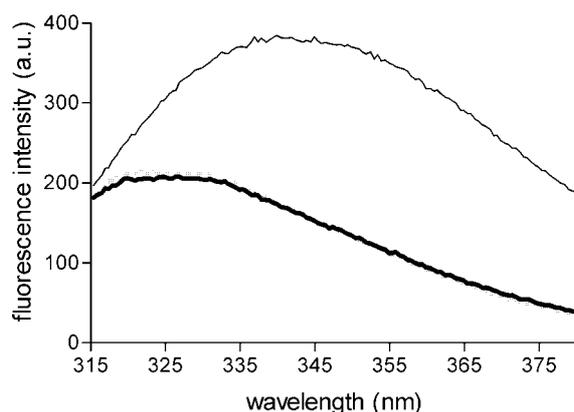


Fig. 3-2. Fluorescence spectra of native BLA (thick black line), of native BLA incubated with 0.3 mM CHAPS (grey line) and of BLA that is displaced from polystyrene nanospheres by 0.3 mM CHAPS and which is subsequently separated from these nanospheres using ultrafiltration (thin black line). The incubation time in the presence of surfactant is 90 minutes. The fluorescence intensities are corrected to compensate for differences in protein concentrations (2-4 μ M). The samples were measured at 20 $^{\circ}$ C in a 4*10 mm quartz cuvette at an excitation wavelength of 300 nm. The calcium concentration is between 1 and 2 mM.

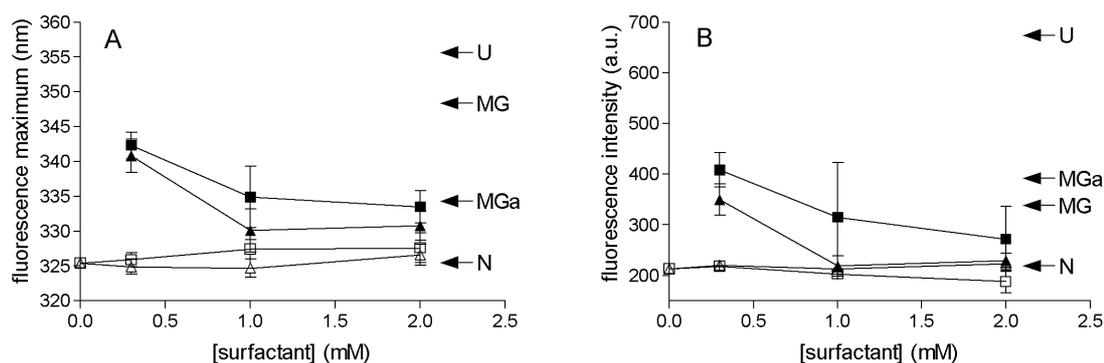


Fig. 3-3. Surfactant concentration dependence of the fluorescence emission maximum (A) and the corresponding fluorescence intensity at this maximum (B) of BLA which is displaced from polystyrene nanospheres by CHAPS (filled triangles) or Tween 20 (filled squares) and which is subsequently separated from these nanospheres using ultrafiltration, and of native BLA incubated with CHAPS (open triangles) and Tween 20 (open squares). The incubation time in the presence of surfactants is 90 minutes. The arrows indicate specific fluorescence properties of the following different conformations of BLA: the native conformation (N), the unfolded conformation (U, as induced by 7.1 M GdnHCl), the MG conformation (MG, induced by adjusting the pH to 2), and the MG-like conformation induced by adsorption of BLA on polystyrene nanospheres (MGa). The error bars are the standard deviations calculated from 3 to 6 individual measurements. The samples were measured at 20 $^{\circ}$ C in a 4*10 mm quartz cuvette at an excitation wavelength of 300 nm. The calcium concentration is between 1 and 2 mM.

The fluorescence emission maximum of BLA displaced by 0.3 mM surfactant shows a red shift of 16 to 18 nm, compared to native BLA (Fig. 3-2). This red shift is larger than the 10 nm red shift observed for adsorbed BLA, but lower than the 23 nm red shift observed for the acid-induced MG state of BLA (Fig. 3-3A). At a CHAPS concentration of 1 and 2 mM, the red shift of displaced BLA is 5 nm compared to native BLA. For

BLA displaced with 1 and 2 mM Tween 20, a less pronounced decrease in red shift of the fluorescence emission maximum is observed.

The fluorescence intensity at the emission maximum of displaced BLA at a surfactant concentration of 0.3 mM is almost twice that of native BLA (Fig. 3-3B). This intensity value is similar to the fluorescence intensity at the emission maximum for the adsorption-induced MG state and the acid-induced MG state of BLA. At higher surfactant concentrations, the fluorescence intensity at the emission maximum of displaced BLA decreases, and in case of 1 and 2 mM CHAPS, the fluorescence intensity at maximum emission becomes similar to that of native BLA. The red shift of the fluorescence emission maximum, and the accompanied increase of the fluorescence intensity, indicates exposure of tryptophan residues in displaced BLA. The intensity increase is related to a reduction in quenching that results from a different position of the tryptophan residues compared to their original position in the three-dimensional structure of native BLA. Control experiments in which native BLA is incubated with different amounts of surfactant show that both the fluorescence emission maximum and the fluorescence intensity at maximum emission are similar to those of the native state (Figs. 3-3A and 3-3B, open symbols). The latter shows that the surfactants themselves do not affect the fluorescence properties of the protein.

Circular dichroism spectra in the far-UV region show that the secondary structure content of all displaced BLA samples equals or exceeds the secondary structure content of native BLA (Figs. 3-4A and 3-4B). BLA displaced by 1 and 2 mM CHAPS has a far-UV CD spectrum that is similar to that of native BLA (Fig. 3-4A). However, at a CHAPS concentration of 0.3 mM, the far-UV CD spectrum is significantly different from that of native BLA. It has a more negative ellipticity between 200 and 230 nm and it has a pronounced minimum around 208 nm (Fig. 3-4A). The latter minimum is characteristic of the far-UV CD spectrum of the acid-induced MG state of BLA. Displacement of BLA by 1 and 2 mM Tween 20 results in far-UV CD spectra that are similar to the one of native BLA (Fig. 3-4B). In contrast to the results observed with 0.3 mM CHAPS, the far-UV CD spectrum of BLA displaced by 0.3 mM Tween 20 is only slightly different from the far-UV CD spectrum of native BLA. Now, the ellipticity between 200 and 230 nm is only slightly more negative than is observed for BLA in the native state. Again, a minimum around 208 nm is observed.

CD spectra in the near-UV region are very sensitive to changes in the microenvironment of aromatic residues. The near-UV CD spectrum of BLA displaced with 0.3 mM CHAPS is similar to the spectrum of BLA in the MG state, indicating the loss of tertiary structure (Fig. 3-5A). At a concentration of 1 and 2 mM CHAPS however, the features of the spectra are similar to those of the spectrum of native BLA, although the ellipticities are reduced compared to the spectrum of native BLA. In the

case of BLA displaced with 0.3 mM and 1 mM Tween 20, the near-UV CD spectra are similar to the spectrum of the acid-induced MG state of BLA (Fig. 3-5B). BLA displaced by 2 mM Tween 20 has a near-UV CD spectrum that shows the presence of tertiary structure, although the intensity of the spectrum is only half that of native BLA.

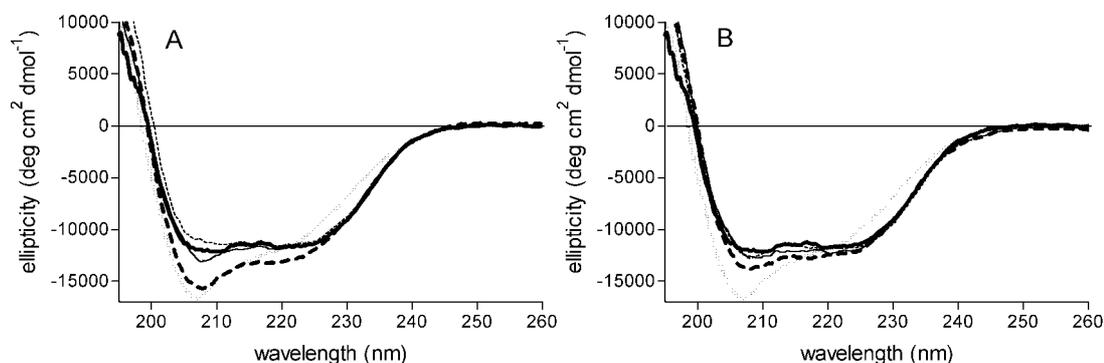


Fig. 3-4. Far-UV CD spectra of native BLA (thick black line), the MG state of BLA at pH 2.1 (grey line), and of BLA that is displaced from polystyrene nanospheres by different amounts of CHAPS (A) or Tween 20 (B) and that is subsequently separated from these nanospheres using ultrafiltration. The surfactant concentrations used to displace BLA are 0.3 mM (thick dashed line), 1 mM (thin black line) and 2 mM (thin dashed line), respectively. The incubation time in the presence of the surfactants is 90 minutes. The protein concentration ranges between 6 and 14 μ M. The samples are measured at 20 °C in a 1 mm quartz cuvette. The calcium concentration is between 1 and 2 mM.

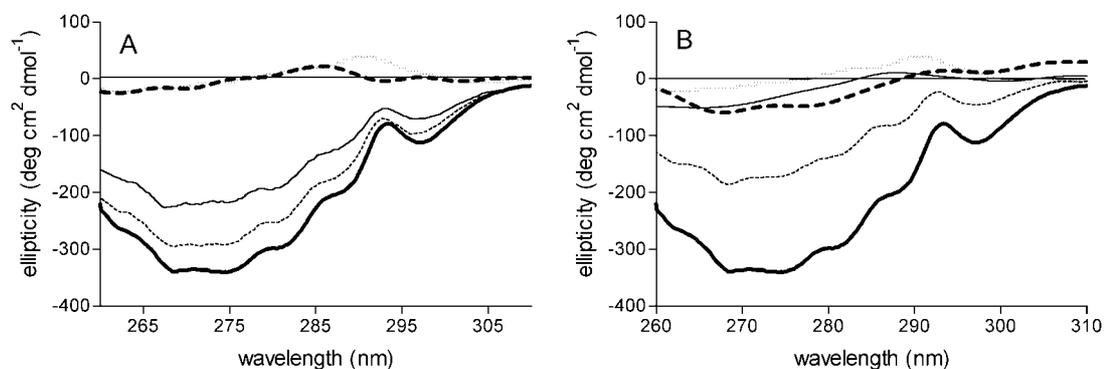


Fig. 3-5. Near-UV CD spectra of native BLA (thick black line), the MG state of BLA at pH 2.1 (grey line), and of BLA that is displaced from polystyrene nanospheres by different amounts of CHAPS (A) or Tween 20 (B) and that is subsequently separated from these nanospheres using ultrafiltration. The surfactant concentrations used to displace BLA are 0.3 mM (thick dashed line), 1 mM (thin solid line) and 2 mM (thin dashed line), respectively. The incubation time in the presence of the surfactants is 90 minutes. The protein concentration ranges between 5 and 20 μ M. The samples are measured at 20 °C in a 10 mm quartz cuvette. The calcium concentration is between 1 and 2 mM.

In control experiments, the effect of both surfactants on native BLA is studied. Both far-UV (Fig. 3-6A) and near-UV (Fig. 3-6B) CD spectra of BLA that is incubated for at least 24 hours with 1 mM Tween 20 or 1 mM CHAPS, are similar to the corresponding

spectra of native BLA. This shows that the surfactant itself does not affect the CD spectrum of BLA.

3.3.3 The non-native properties of BLA displaced by 0.3 mM surfactant

The intrinsic fluorescence and CD spectra discussed above indicate that BLA that is displaced from a polystyrene interface by 1 or 2 mM surfactant has native-like properties. The spectra also show that displacement by 0.3 mM surfactant results in displaced BLA molecules with non-native properties. To obtain insight in the causes for this observation, three additional displacement experiments and non-denaturing gel electrophoresis experiments are done using CHAPS as the surfactant.

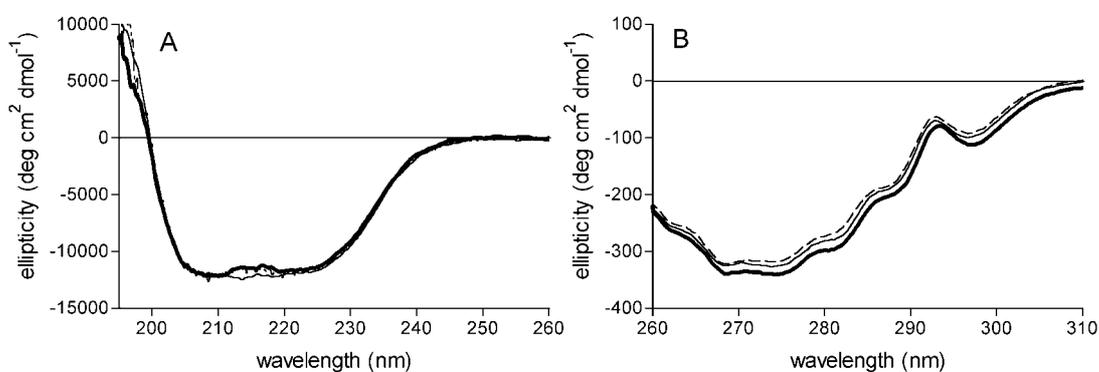


Fig. 3-6. Far-UV (A) and near-UV (B) CD spectra of native BLA (thick black line), of BLA incubated for at least 24 hrs with 1 mM Tween 20 (thin black line), and of BLA incubated for at least 24 hrs with 1 mM CHAPS (dashed line). The protein concentration ranges between 5 and 10 μ M for far-UV CD measurements and is approximately 20 μ M for near-UV CD measurements. The samples are measured at 20 °C in a 1 mm quartz cuvette (far-UV) or in a 10 mm quartz cuvette (near-UV). The calcium concentration is 1 mM.

In the first displacement experiment, the incubation time, during which displacement of adsorbed BLA by 0.3 mM CHAPS occurs, is varied. After an incubation time of 20 minutes, the fluorescence spectrum of BLA displaced by 0.3 mM CHAPS is only slightly altered compared to the spectrum of native BLA (Fig. 3-7). After an incubation time of 16 hours however, the spectrum is red-shifted and the fluorescence intensity is increased. A similar observation is made after an incubation time of 90 minutes, the minimum incubation time required to observe non-native properties in displaced BLA (Fig. 3-2). The conformational changes observed at 0.3 mM CHAPS are thus time-dependent.

In the second displacement experiment, adsorbed BLA is incubated during 16 hours with 0.3 mM CHAPS. Subsequently, additional CHAPS is added to a final concentration of 2 mM to increase the amount of BLA molecules displaced from the polystyrene nanospheres. Ultrafiltration results in a recovery of 62% of the initial

amount of adsorbed BLA molecules. This is lower than the 90% recovery obtained after direct incubation of adsorbed BLA with 2 mM CHAPS for 16 hours. This observation indicates the presence of BLA molecules tightly bound to or possibly covalently bound to the polystyrene nanospheres. The corresponding fluorescence spectrum of this sample again shows the presence of displaced BLA with non-native properties (Fig. 3-8). In fact, the fluorescence spectrum is more red shifted and has a higher fluorescence intensity than observed in the spectrum of BLA displaced by 0.3 mM CHAPS after the same incubation time (16 hours). This shows that BLA in the adsorbed state also has non-native conformational properties after incubation for 16 hours with 0.3 mM CHAPS. However, incubation of adsorbed BLA with 2 mM CHAPS for 16 hours, results in a fluorescence spectrum of displaced BLA (90% recovery) that is almost native-like (Fig. 3-8).

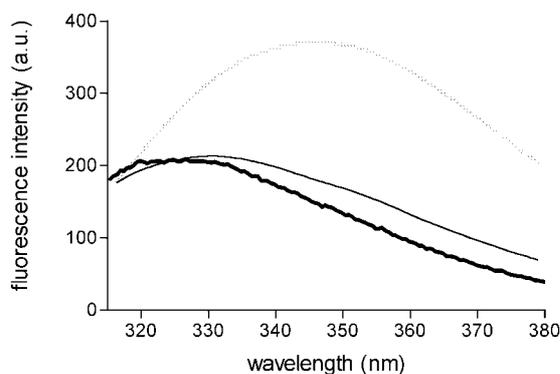


Fig. 3-7. Fluorescence spectra of BLA displaced from polystyrene nanospheres by incubation with 0.3 mM CHAPS at different incubation times. Native BLA (thick black line); displaced BLA after 20 minutes of incubation (thin black line); displaced BLA after 16 hours of incubation (thick grey line). The fluorescence intensities are corrected to compensate for differences in protein concentrations (2 - 4 μ M). The samples are measured at 20 °C in a 4*10 mm quartz cuvette at an excitation wavelength of 300 nm. The calcium concentration is between 1 and 2 mM.

In the third displacement experiment, the persistence of the observed non-native properties of BLA displaced by 0.3 mM CHAPS is investigated. First, BLA is displaced from polystyrene nanospheres by 0.3 mM CHAPS for a period of 16 hours, followed by additional displacement with 2 mM CHAPS. Subsequently, this displaced BLA is unfolded by the addition of GdnHCl to a final concentration of 5.3 M. This denaturant concentration suffices to unfold native BLA. Next, BLA is forced to refold by diluting the sample with buffer to a final GdnHCl concentration of 0.9 M, a denaturant concentration at which BLA is in the native state. The corresponding fluorescence spectrum shows that the native conformation is not recovered. In fact, refolded displaced BLA has the same non-native fluorescence properties as BLA displaced by

0.3 mM CHAPS after an incubation time of 16 hours (Fig. 3-8). The fluorescence spectrum of native BLA at a concentration of 0.9 M GdnHCl however, has the same fluorescence emission maximum, and a little lower fluorescence intensity compared to the fluorescence spectrum of native BLA in the absence of GdnHCl. Clearly, the displacement of BLA from polystyrene nanospheres by 0.3 mM CHAPS results in conformational changes which are recovered upon protein unfolding and subsequent refolding.

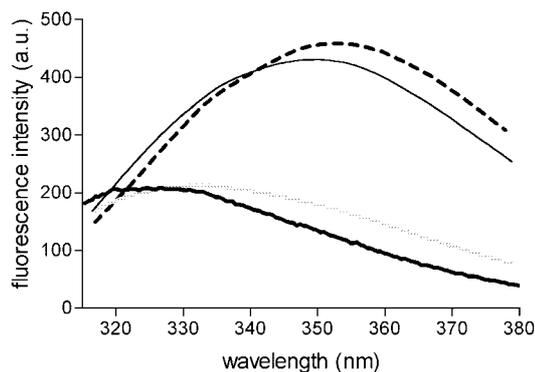


Fig. 3-8. Fluorescence spectra of BLA displaced from polystyrene nanospheres by CHAPS using different procedures. Native BLA (thick black line); displaced BLA after 16 hours of incubation with 0.3 mM CHAPS and subsequent incubation with 2 mM CHAPS (thin black line); displaced BLA after 16 hours of incubation with 2 mM CHAPS (thick grey line); displaced BLA after 16 hours of incubation with 0.3 mM CHAPS and subsequent incubation with 2 mM CHAPS, after which the displaced BLA is unfolded by 5.3 M GdnHCl and again refolded by diluting the denaturant to 0.9 M GdnHCl (dashed line). The fluorescence intensities are corrected to compensate for differences in protein concentrations between the samples (2 - 4 μ M). The samples are measured at 20 °C in a 4*10 mm quartz cuvette at an excitation wavelength of 300 nm. The calcium concentration is between 1 and 2 mM.

Finally, in non-denaturing gel electrophoresis experiments, significant differences are observed between the electrophoretic mobility of native BLA and BLA displaced from polystyrene nanospheres by 0.3 mM CHAPS (Fig. 3-9). Fig. 3-9A illustrates the effect of the removal of both calcium ions of holo-BLA by the addition of EDTA. Apo-BLA has a higher electrophoretic mobility on a non-denaturing gel than holo-BLA due to its increased negative charge compared to holo-BLA. The effect of adsorption of BLA on polystyrene nanospheres and its subsequent displacement by 0.3 mM CHAPS is shown in Fig. 3-9B (lanes 2, 3 and 4). Three additional bands of displaced BLA molecules with higher electrophoretic mobilities than apo-BLA are observed. These additional bands are not observed for BLA that is displaced from the polystyrene interface by 1 and 2 mM CHAPS, respectively. Apparently, the non-native properties detected by fluorescence and CD spectroscopy for the ensemble of BLA molecules displaced by 0.3 mM CHAPS, are associated with the presence of a population of

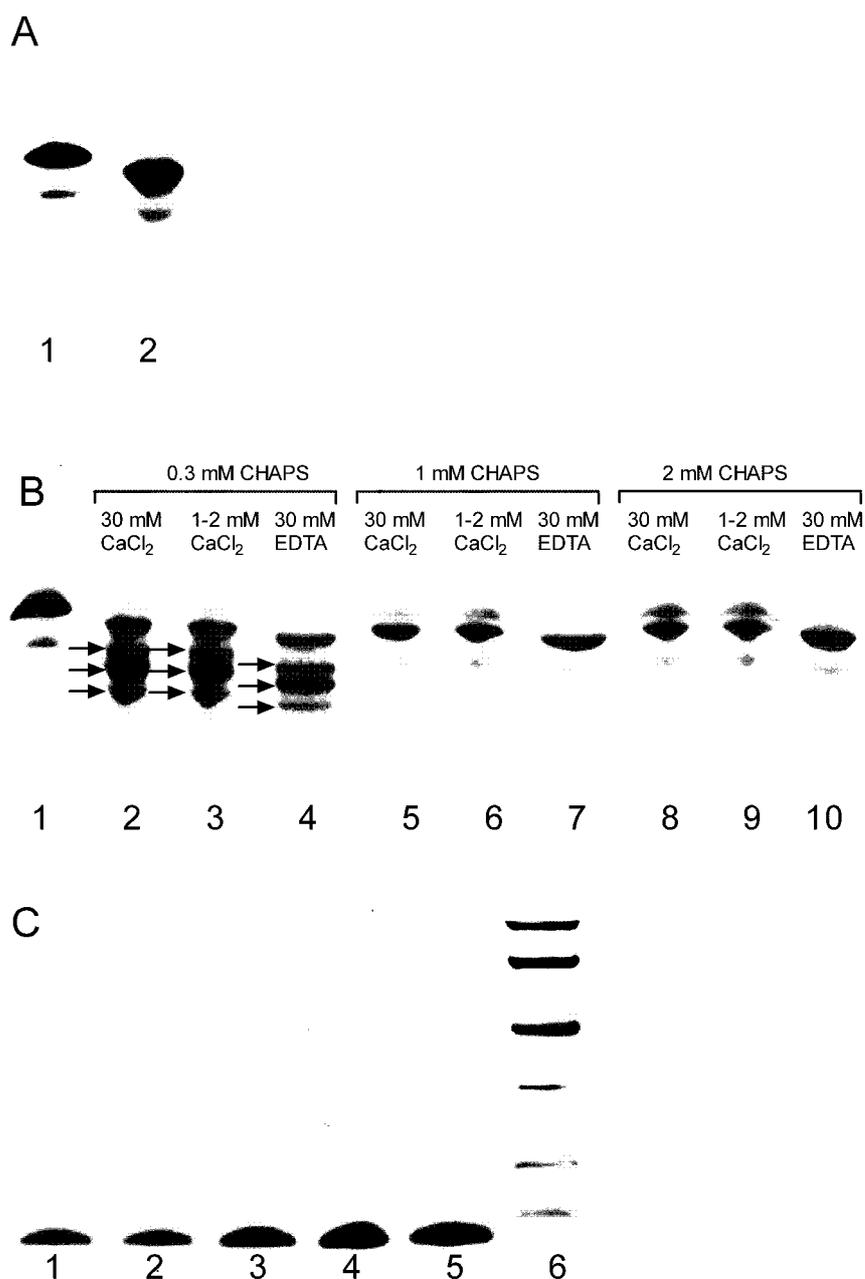


Fig. 3-9. Polyacrylamide gel electrophoresis (PAGE) of BLA that is displaced from polystyrene nanospheres by different concentrations of CHAPS in the presence and absence of calcium. Non-denaturing gel (A) of BLA in the presence of 30 mM CaCl₂ (lane 1) and BLA in the presence of 30 mM EDTA (lane 2). The tiny band observed below the main BLA band is due to an unknown minor component in Sigma BLA. Non-denaturing gel (B) of BLA in the presence of 30 mM CaCl₂ (lane 1), and of BLA displaced by 0.3 mM CHAPS (lanes 2-4), by 1.0 mM CHAPS (lanes 5-7) and by 2.0 mM CHAPS (lanes 8-10); 30 mM CaCl₂ is added to the samples in lanes 2, 5 and 8; 30 mM EDTA is added to the samples in lanes 4, 7 and 10; 1 – 2 mM CaCl₂ is present in lanes 3, 6, and 9. The arrows indicate bands of non-native BLA molecules that have a higher electrophoretic mobility than native BLA. SDS-PAGE (C) of BLA displaced from polystyrene nanospheres by 0.3 mM CHAPS (lane 1), by 1.0 mM CHAPS (lane 2) and by 2.0 mM CHAPS (lane 3); of BLA in the presence of 30 mM CaCl₂ (lane 4), of BLA in the presence of 30 mM EDTA (lane 5), and of marker proteins (lane 6). The molecular weights of the markers proteins are: 94, 67, 43, 30, 20.1 and 14.4 kDa. For all gels, the direction of migration is down, towards the positive electrode on the bottom side of the gel.

displaced BLA molecules that have a higher electrophoretic mobility than native BLA. The high electrophoretic mobility of these displaced BLA molecules with non-native properties could be caused by a different charge distribution compared to native BLA. Addition of 30 mM calcium does not affect the mobility of displaced BLA (lanes, 2, 5 and 8 in Fig. 3-9B). In contrast, addition of 30 mM EDTA causes the increase of the electrophoretic mobility of all bands, which shows that displaced BLA molecules contain calcium (lanes 4, 7 and 10 in Fig. 3-9B). The SDS gel shows that the higher electrophoretic mobility of the three additional bands is not caused by the presence of protein fragments due to cleavage of BLA (Fig. 3-9C).

3.3.4 *The role of calcium*

In chapter 2 it is shown that BLA loses calcium during adsorption on polystyrene nanospheres (Engel, et al., 2002). Here, it is shown that BLA that is displaced from the interface again contains calcium (Fig. 3-9B). BLA that is displaced by 1 and 2 mM CHAPS has two main bands on a non-denaturing gel (lanes 5, 6, 8 and 9 in Fig. 3-9B). The slowest migrating band of these two is assigned to displaced BLA in which both calcium binding sites are occupied. The faster migrating band, which corresponds to the majority of the displaced BLA molecules, is assigned to displaced BLA in which the calcium binding site with the highest affinity is occupied. Association of a calcium ion with the second binding site, which has a lower affinity, is apparently a slow process that was not completed at the time the gel was run. Hence, two bands are observed for BLA that is displaced by 1 and 2 mM CHAPS. Addition of EDTA should lead to the merging of these bands at the position expected for apo-BLA, as is indeed observed (lanes 7 and 10 in Fig. 3-9B).

To further support the presence of calcium in displaced BLA, the calcium concentration of several samples containing BLA, or nanospheres, or CHAPS, or combinations of these, are determined by ICP-OES (Table 3-1). A calcium-free buffer is used, in contrast to the previous experiments. The calcium concentrations are determined in samples that are first subjected to ultrafiltration. Hence, the samples do not contain nanospheres. The following observations are made. Firstly, Table 3-1 shows that the buffer, the nanospheres, and CHAPS contain a negligible amount of calcium. Secondly, the sample that contains approximately 35 μM BLA by dissolving freeze-dried BLA (Sigma) has a calcium concentration of 72 μM . Consequently, both calcium binding sites are occupied in BLA obtained from Sigma, as is supported by the non-denaturing gel electrophoresis experiments (Fig. 3-9B). Thirdly, after incubation of CaCl_2 with nanospheres, only a small amount of calcium can be detected in solution. Fourthly, after BLA is incubated with nanospheres, the amount of calcium in the filtrate equals the molar amount of BLA that is not adsorbed. Fifthly, after displacement of

adsorbed BLA by 1 mM CHAPS, the amount of calcium in the filtrate again equals the amount of displaced BLA. These measurements confirm that calcium ions adsorb on the polystyrene nanospheres (Engel, et al., 2002). The measurements on the samples containing both BLA and nanospheres show that the molar amount of calcium detected in solution equals the molar amount of BLA that is not adsorbed. These non-adsorbed BLA molecules thus still contain calcium (Table 3-1). Finally, after displacement of almost half of the amount of adsorbed BLA by 1 mM CHAPS, the molar amount of calcium in solution equals the molar amount of displaced BLA. This shows that the majority of the displaced BLA molecules contains a single calcium ion, as is also observed in native gel electrophoresis experiments (Fig. 3-9B).

Table 3-1. Determination of the concentration of calcium and/or BLA in various samples that are subjected to ultrafiltration to remove polystyrene nanospheres.^a

| Samples ^b | [Calcium] (μM) | n ^c | [BLA] (μM) | m ^d |
|---|-----------------------------|----------------|-------------------------|----------------|
| buffer (10 mM Tris/HCl pH 7.5) | 0.5 ± 0.3 | 3 | n.d. | - |
| buffer + 1 mM CHAPS | 0.7 ± 0.5 | 3 | n.d. | - |
| 100 nM NS ^e | 0.4 ± 0.5 | 2 | n.d. | - |
| 40 μM CaCl ₂ | 39.1 ± 0.6 | 3 | n.d. | - |
| 20 μM CaCl ₂ + 80 nM NS | 3.0 ± 0.4 | 2 | n.d. | - |
| 40 μM CaCl ₂ + 80 nM NS | 2.7 ± 0.4 | 2 | n.d. | - |
| 80 μM CaCl ₂ + 80 nM NS | 6.1 ± 0.5 | 2 | n.d. | - |
| 40 μM BLA | 72.3 ± 2.1 | 2 | 34.6 ± 0.2 | 2 |
| 40 μM BLA + 80 nM NS | 6.3 ± 0.9 | 3 | 5.9 ± 0.5 | 3 |
| 40 μM BLA + 100 nM NS | 3.6 ± 1.2 | 2 | 3.5 ± 0.3 | 2 |
| 40 μM BLA + 100 nM NS + 1 mM CHAPS | 17.3 ± 1.3 | 3 | 17 ± 1 | 3 |

^a The error margins represent the standard deviations, calculated from two or three individual measurements.

^b All samples were measured in a calcium free 10 mM Tris/HCl buffer at pH 7.50. All samples, except “buffer + 1 mM CHAPS” were filtrated using an Amicon 50 kD ultrafiltration device before determination of the calcium concentration and/or the BLA concentration.

^c n is the number of individual determinations of the calcium concentration

^d m is the number of individual determinations of the BLA concentration.

^e NS = nanospheres

3.4 Discussion

Bovine α -lactalbumin molecules that are displaced from a hydrophobic interface by a surfactant should regain the native three-dimensional structure of BLA as the forces that induce an MG-like state of BLA on such an interface are absent after displacement. However, the presented results indicate that this is only partly the case. The conformational properties of displaced BLA molecules, as determined by intrinsic

fluorescence spectroscopy, CD spectroscopy, and non-denaturing gel electrophoresis, depend on the concentration of the surfactant used to displace BLA and on the incubation time during which displacement takes place.

At a high (1 - 2 mM) surfactant concentration, displaced BLA has native-like CD and fluorescence properties. This indicates that the adsorption-induced conformational changes of BLA are reversible upon displacement. BLA adsorbed on hydrophobic polystyrene nanospheres is in an MG-like state and does not contain calcium (Engel, et al., 2002). Upon addition of 2 mM CHAPS, 90% of the adsorbed BLA molecules is displaced and these molecules subsequently refold to holo-BLA with native-like fluorescence and CD properties. Our displacement studies show that CHAPS does not affect the conformation of native BLA, which is in agreement with previous studies (Womack, et al., 1983).

Displacement of BLA by low surfactant concentrations (0.3 mM CHAPS or Tween 20) at long incubation times results in BLA samples which have non-native spectral properties. BLA incubated with 0.3 mM CHAPS is characterized by a 16 nm red shifted fluorescence emission maximum, by a twofold increase in the fluorescence maximum intensity compared to native BLA, by a far-UV CD spectrum that is native-like, by a strongly decreased ellipticity in the near-UV CD spectrum compared to native BLA and by the presence of a population of BLA molecules that have a higher electrophoretic mobility than native BLA. The results show that these non-native spectral properties can not be caused by insufficiently available calcium (Table 3-1 and Fig. 3-9).

It is proposed that the non-native properties of displaced BLA molecules are caused by a chemical reaction of adsorbed BLA with a few reactive groups on the surface of the polystyrene nanospheres. The fraction of non-native molecules in the ensemble of displaced BLA molecules is increased by the following process. At a CHAPS concentration of 0.3 mM, only 15% of the BLA molecules are displaced from the surface, while 85% of the BLA molecules are attached to the nanospheres. Displaced BLA molecules are able to exchange with adsorbed BLA molecules under these conditions. A constant homomolecular exchange between adsorbed and displaced molecules is taking place. It is this homomolecular exchange of adsorbed and displaced BLA molecules, in combination with the presence of reactive groups on the surface of the polystyrene nanospheres, that causes the time-dependent non-native properties of BLA displaced at a surfactant concentration of 0.3 mM. After 16 hours of exchange at a surfactant concentration of 0.3 mM CHAPS, the spectral properties of the 15% displaced BLA molecules are similar to those of the remaining 85% adsorbed BLA molecules once they have been nearly completely displaced from the interface by incubation with 2 mM CHAPS (Fig. 3-8). This is to be expected when homomolecular exchange is occurring at a sufficient rate: a similar fraction of adsorbed and displaced

BLA molecules will have reacted with reactive groups on the polystyrene nanospheres. At a CHAPS concentration of 2 mM however, the majority of the BLA molecules is displaced, and the surfactant molecules cover most of the polystyrene interface. As a consequence, homomolecular exchange of BLA molecules is greatly reduced, and indeed, virtually no non-native properties of displaced BLA molecules are observed.

What is the likely nature of the chemical reaction occurring with adsorbed BLA? The event happening with a fraction of the adsorbed BLA molecules on the polystyrene surface is such that it is not reversed upon separation of BLA from the nanospheres. These displaced BLA molecules do not refold to native BLA. Unfolding of these BLA molecules by a high concentration of GdnHCl and subsequent refolding by lowering the concentration denaturant does not lead to native BLA, instead the non-native properties are reproduced (Fig. 3-8). As the observed changes are permanent and independent of the type of surfactant used (i.e. CHAPS and Tween 20), the only plausible cause is intramolecular disulfide shuffling of adsorbed BLA molecules due to the presence of reactive groups at the polystyrene interface. The reactive groups might be remainders of initiators or styrene monomers needed during the production of polystyrene nanospheres. The resulting non-native disulfide variants of BLA cannot form native BLA upon unfolding by GdnHCl and subsequent refolding. The presence of these non-native disulfide variants of BLA explains the distinct additional bands in the non-denaturing gels discussed before. The corresponding MG-like properties observed by fluorescence and CD spectroscopy can be compatible with a variety of disulfide bonds pairings in these non-native disulfide variants of BLA (Ewbank and Creighton, 1991). Differences in shape and charge distribution of these disulfide shuffled BLA variants compared to native BLA explains their relatively high electrophoretic mobilities.

Only a few reactive groups need to be present on the polystyrene nanospheres for intramolecular disulfide shuffling in adsorbed BLA molecules to occur. As long as the incubation time is sufficient, a significant fraction of the BLA molecules will have been homomolecularly exchanged and been in contact with these reactive groups. This causes the observed phenomena described above. The reactive groups on the polystyrene nanospheres appear not to be sulfhydryl groups, as they were not detected using Ellman's reagent.

In case of homomolecular exchange of bovine serum albumin adsorbed on a polystyrene interface, it is proposed that the observed irreversible conformational changes are caused by aggregate formation of the exchanged protein molecules (Norde, et al., 2000). This is not the case for BLA adsorption and displacement described here. Gel filtration experiments (data not shown) and non-denaturing gel electrophoresis experiments (Fig. 9B) show that no aggregation of displaced BLA is taking place.

The results show that the surfactants Tween 20 and CHAPS are able to displace adsorbed BLA from the surface of polystyrene nanospheres, although their effectiveness in doing so differs. The latter must be explained by differences in the properties of both surfactants. Tween 20 is a nonionic surfactant with a critical micelle concentration (CMC) of 0.059 mM in water (Helenius, et al., 1979), while CHAPS is a zwitterionic surfactant with a CMC of 5 mM in a 10 mM phosphate buffer at pH 8 (Giacomelli, et al., 2000). The Tween 20 concentrations used in the experiments here are much higher than its CMC. Thus, most Tween 20 molecules form micelles, which might be one of the causes for this surfactant being less effective in displacing BLA from the polystyrene nanospheres as compared to CHAPS. In addition, since CHAPS molecules are zwitterionic they can have an additional, electrostatic interaction with the slightly negatively charged polystyrene nanospheres as opposed to Tween 20, making them more effective in BLA displacement.

The protein displacement observations described in this paper are limited to one protein (BLA), one surface (polystyrene nanospheres) and two surfactants (CHAPS and Tween 20). It has been shown before that a change of surface, for example replacing a hydrophobic surface by a hydrophilic surface, results in different adsorption behaviour, which may lead to differences in the conformation of the adsorbed protein. However, the effectiveness of protein displacement by the specific surfactants may be generalized. The interaction energy between BLA and the hydrophobic interface is expected to be large, based on the very fast kinetics of the adsorption process (Engel, et al., 2002). Despite the strong interaction, both Tween 20 and CHAPS are able to displace BLA from a hydrophobic surface. Therefore, both surfactants – and in particular CHAPS – are good candidates for the removal of proteins from surfaces in general. The generation of displaced protein molecules in their native state is an advantage of the use of these specific surfactants.

The presented procedure of protein adsorption and subsequent displacement by a surfactant is relevant for the study of protein conformations on interfaces. The detailed study of the conformational characteristics of adsorbed BLA using NMR spectroscopy in combination with H/D exchange experiments is envisaged. Although direct information about the properties of proteins at interfaces can not easily be obtained using NMR spectroscopy, indirect information can be extracted. Amide hydrogens of BLA adsorbed on an interface are allowed to exchange with the deuterated solvent. The exchange characteristics inform about the local stability of the adsorbed protein and about the parts of the protein that are solvent inaccessible due to adsorption. The adsorbed protein molecules need to be displaced from the interface to be able to benefit from the power of modern high-resolution NMR measurements in solution. Lowering the pH of the solution simultaneously during displacement quenches hydrogen

exchange. If the protein is in the native state after displacement and full resonance assignments are available, as is the case for BLA, then it is straightforward to detect the exchange of individual amide hydrogens of the previously adsorbed protein and detect conformational characteristics of the adsorbed protein. A necessity for this method is the ability to displace the protein from the surface to which it is adsorbed and, preferably, the displaced protein should be in the native state. Here, it is demonstrated that both requirements are fulfilled for BLA adsorbed on polystyrene nanospheres using CHAPS as displacing surfactant. A solid basis is thus laid for the future detailed characterization of the conformation of adsorbed BLA.

3.5 Conclusions

This study shows that adsorbed BLA, which is in a partially unfolded state on a hydrophobic interface, can be displaced and refolded to BLA with native properties by the use of a surfactant. However, at low surfactant concentrations and prolonged incubation times, displaced BLA has non-native properties. This displaced BLA is characterized by a red shifted fluorescence emission maximum, by a twofold increase in the fluorescence intensity as compared to native BLA, by a native-like far-UV CD spectrum, by a strongly decreased ellipticity in the near-UV CD spectrum as compared to native BLA and by the presence of a population of BLA molecules that has a higher electrophoretic mobility than native BLA. These non-native properties can be caused by intramolecular disulfide shuffling initiated through the presence of a few reactive groups on the polystyrene nanosphere surface, during homomolecular exchange of proteins at low (0.3 mM) surfactant concentration. At high (1 – 2 mM) surfactant concentrations, homomolecular exchange does not play a significant role, hence displaced BLA has native-like properties under these conditions. Finally, the displacement procedure presented here enables the future detailed characterization of the conformation of proteins on interfaces.

4 Adsorption of bovine α -lactalbumin on suspended solid nanospheres and its subsequent displacement studied by NMR spectroscopy

Detailed knowledge of the adsorption-induced conformational changes of proteins is essential to understand the process of protein adsorption. However, not much information about these conformational changes is available. Here, the adsorption of apo- and holo-bovine α -lactalbumin (BLA) on suspended solid polystyrene nanospheres and their subsequent displacement by a surfactant are studied by NMR spectroscopy. To my knowledge, this is the first time that adsorption of proteins on solid nanospheres, with both components present in the NMR sample, is studied by this method. High quality 1D and 2D ^1H -NMR spectra of non-adsorbed apo- and holo-BLA in the presence of BLA- or surfactant-covered solid polystyrene nanospheres in suspension are obtained using standard NMR procedures. BLA and surfactant molecules that are adsorbed on the polystyrene nanospheres give rise to extremely broadened proton resonances. This can be exploited to determine the amount of adsorbed protein and of adsorbed surfactant in a system containing protein, nanospheres and surfactant, without disturbing the equilibrium of the system. Two-dimensional ^1H -NMR spectroscopy shows that the chemical shifts of the backbone amide protons of holo-BLA after its adsorption and subsequent displacement from polystyrene nanospheres by the surfactant CHAPS are identical to those of native holo-BLA. The adsorption-induced unfolding of BLA to a molten globule state on polystyrene nanospheres is thus fully reversible at the residue-level upon CHAPS-induced displacement of BLA. The latter is the now fulfilled essential requirement that enables the future indirect study, at the residue-level, of the conformational characteristics of BLA adsorbed on polystyrene nanospheres by hydrogen/deuterium exchange and NMR spectroscopy. The results presented show that NMR spectroscopy is clearly feasible to study the adsorption of BLA on suspended polystyrene nanospheres. This technique should be applicable to the study of the adsorption of other proteins on other surfaces as well.

4.1 Introduction

The adsorption of protein molecules on interfaces is a ubiquitous phenomenon in both natural and man-made systems. It is now recognized that detailed knowledge of the adsorption-induced conformational changes is essential for understanding the process of protein adsorption. However, not much information about these conformational changes is available, and it is limited to information about the amount of secondary structure in adsorbed proteins, the thickness of the adsorbed protein monolayer, and to the conformational properties of a single, e.g. fluorescently labelled, residue in an adsorbed protein. Detailed structural information at the atomic level about adsorbed proteins is lacking.

Three-dimensional structures of protein molecules are generally obtained using the well-established methods of X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy. However, the suitability of these techniques to study adsorbed proteins is severely limited by experimental difficulties, which are mainly due to the presence of a solid adsorbent. X-ray crystallography requires protein crystals and is thus not suitable for the study of the structure of adsorbed proteins, unless these adsorbed proteins form a two-dimensional crystal, which is very difficult to achieve (Hasler, et al., 1998). NMR spectroscopy has been scarcely used to study the conformation of adsorbed proteins. Limited information about the conformation of adsorbed proteins is obtained by solid-state NMR spectroscopy (Drobny, et al., 2003; Mayer, 2002). The relaxation rates of solvent protons near adsorbed proteins has been studied in solution by NMR spectroscopy (Benko, et al., 1975). The conformation of adsorbed small peptides, composed of up to 14 amino acid residues, could be determined in solution by NMR spectroscopy (Burkett, et al., 2001). In addition, NMR spectroscopy has been used in combination with hydrogen/deuterium (H/D) exchange to study in an indirect way the properties of previously adsorbed proteins after their removal from the interface (Gorenstein, et al., 1994; Keire, et al., 1992; McNay, et al., 2001a; McNay, et al., 2001b).

To my knowledge, NMR spectroscopic studies in solution of protein molecules adsorbed on a solid interface have not been reported. This is not surprising since the motions of the required solid particles are on an undesirable time scale that leads to severe line broadening of the NMR signal of the adsorbed protein. However, the latter phenomenon can be favourably exploited as is shown in this chapter. In addition, NMR spectroscopists usually avoid samples that contain suspended particles since the expected inhomogeneity of these samples would cause magnetic field differences that

would lead to disturbed solution NMR spectra of proteins. The latter is shown here not to be the case for suspended solid polystyrene nanospheres.

In this study, the adsorption of apo- and holo-bovine α -lactalbumin (BLA) on solid polystyrene nanospheres is studied in solution by NMR spectroscopy. BLA is chosen to study protein adsorption because its structure, stability, and folding behaviour have been thoroughly investigated (Chrysina, et al., 2000; Kuwajima, 1996; Pike, et al., 1996; Wijesinha-Bettoni, et al., 2001). In addition, the adsorption of BLA on a variety of interfaces has been studied (Haynes, et al., 1995; Norde, et al., 1992a; Norde, et al., 1995; Oroszlan, et al., 1990; Suttiaprasit, et al., 1992). In chapter 2, it is shown by CD and fluorescence spectroscopy that BLA molecules that are adsorbed on hydrophobic polystyrene nanospheres partially unfold and adopt a molten globule-like conformation (Engel, et al., 2002). This molten globule-like conformation is characterized by the presence of native secondary structure and the absence of persistent tertiary structure.

Here, it is shown that high quality one-dimensional (1D) and two-dimensional (2D) ^1H -NMR spectra of non-adsorbed BLA in the presence of suspended solid polystyrene nanospheres can be obtained using standard NMR procedures. Furthermore, 2D ^1H -NMR spectroscopy is used to study the conformation of BLA after its displacement from polystyrene nanospheres using the surfactant 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The displaced BLA molecules were previously shown to have native-like CD and fluorescence spectral properties (Engel, et al., 2003). It is shown here by NMR spectroscopy that the amide protons of displaced BLA resonate at identical positions as the corresponding protons of native BLA. BLA thus refolds to the native state after its displacement from the nanospheres. The study presented shows that the requirements are fulfilled for the detailed study by H/D exchange and NMR spectroscopy of BLA adsorbed on polystyrene nanospheres.

4.2 Materials and methods

4.2.1 Materials

Bovine α -lactalbumin (L-5385), with approximately 2 moles calcium ions per mole BLA (Engel, et al., 2003), calcium-depleted bovine α -lactalbumin (L-6010), EDTA (E-9884) and CHAPS (C-5070) were obtained from Sigma. Deuterium oxide, 99.9% was obtained from Cambridge Isotope Laboratories, Inc. (USA). Polystyrene nanospheres, supplied as a concentrated 7.9% (w/w) colloidal suspension in water, were obtained from Interfacial Dynamics Corporation (Portland, USA). The radius of the nanospheres is 60 ± 1 nm as determined by dynamic light scattering. A 20 mM sodium acetate buffer at pH 5.9 and 20 mM sodium phosphate buffers at pH 6.0, pH 7.0, and pH 7.4 were

used in the experiments. Nanopure water (Sybron Barnstead NANOpure II) was used as a solvent for the preparation of the samples.

4.2.2 Adsorption and displacement of BLA studied by NMR spectroscopy

Small volumes of a solution of 8.5 mM holo-BLA in 20 mM sodium acetate at pH 5.7 were added with a Hamilton syringe to an NMR tube containing 550 μ l of 123 nM polystyrene nanospheres in 20 mM sodium acetate at pH 5.9. The final holo-BLA concentrations were 0.11 mM, 0.21 mM, 0.31 mM and 0.41 mM, respectively, and the final pH was 5.9. These samples were used for acquiring 1D 1 H-NMR spectra at 35 $^{\circ}$ C. The 123 nM polystyrene nanosphere suspension was made by mixing 482 μ l of 7.9% (w/w) polystyrene nanospheres, 1.2 μ l of 1 M 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS), 12 μ l of a 1.0 M sodium acetate buffer at pH 5.7, and 55 μ l 2 H $_2$ O. The sample of displaced holo-BLA was made by the addition of 18 μ l of a 0.64 M solution of CHAPS to the NMR tube containing adsorbed holo-BLA with a final protein concentration of 0.41 mM (see above), which results in a final CHAPS concentration of 19 mM. This sample was used for acquiring a 1D 1 H-NMR spectrum and a 2D 1 H-TOCSY NMR spectrum, both at 35 $^{\circ}$ C.

The adsorption of apo-BLA on polystyrene nanospheres was studied similarly. Small volumes of a solution of 8.0 mM apo-BLA in 20 mM sodium phosphate pH 7.0 containing 5 mM EDTA were added with a Hamilton syringe to an NMR tube containing 550 μ l of 123 nM polystyrene nanospheres in 20 mM sodium phosphate at pH 7.4. The resulting final apo-BLA concentrations were 0.22 mM, and 0.42 mM, respectively, and the final pH was 7.4. These samples were used for acquiring 1D 1 H-NMR spectra at 24 $^{\circ}$ C. The 123 nM polystyrene nanosphere solution was made by mixing 482 μ l of 7.9% (w/w) polystyrene nanospheres, 1.2 μ l of 1 M DSS, 12 μ l of a 1.0 M sodium phosphate buffer at pH 7.0, and 55 μ l 2 H $_2$ O. The sample of displaced apo-BLA was made by the addition of 9 μ l of a 0.64 M solution of CHAPS to the NMR tube containing adsorbed apo-BLA with a final protein concentration of 0.42 mM (see above), which results in a final CHAPS concentration of 10 mM. This sample was used for acquiring a 1D 1 H-NMR spectrum at 24 $^{\circ}$ C.

After each addition of either holo- or apo-BLA or CHAPS to the nanospheres suspension, the contents of the NMR tube were carefully mixed by inverting the tube several times, followed by short centrifugation of the tube to force the contents to the bottom of the NMR tube.

4.2.3 NMR spectroscopy

All NMR experiments were recorded at 35 $^{\circ}$ C (unless mentioned otherwise) on a Bruker AMX500 equipped with a triple resonance 5 mm inverse probe. Protein

containing samples were prepared from a filtered (0.2 μm , Schleicher & Schuell GmbH, Germany) stock solution of 8.0 to 8.5 mM BLA. The BLA concentration of the samples ranges from 0.11 to 0.60 mM. The concentration of BLA was measured spectrophotometrically using a molar extinction coefficient of $28,540 \text{ M}^{-1}\text{cm}^{-1}$ for BLA at 280 nm. All samples contain 10% $^2\text{H}_2\text{O}$. Cross peaks were assigned using total correlation spectroscopy (TOCSY), double quantum filtered correlation spectroscopy (DQF-COSY) and nuclear Overhauser effect spectroscopy (NOESY) 2D ^1H -NMR spectra, and using reported assignments (Forge, et al., 1999). The 2D ^1H -TOCSY spectra were acquired using a mixing time of 40 ms with 2048 complex points in t_2 and 350 complex points in t_1 . The number of scans used to acquire the 2D ^1H -TOCSY spectra was 96 for the sample of holo-BLA with 41 mM CHAPS, 160 for the sample of holo-BLA at pH 7.0, 128 for the sample of displaced BLA with 123 nM nanospheres and 19 mM CHAPS, and 192 for the sample of holo-BLA at pH 6.0. The spectral widths were 8064 Hz in both t_2 and t_1 .

A 1D version of the 3-pulse NOESY pulse sequence and phase cycle was used to collect the 1D NMR data; the value of the incremental delay t_1 was fixed at 3 μs , and t_m was kept very short (5 ms). All 1D ^1H -NOESY spectra were acquired with 4096 complex points and 1216 scans, except those acquired of samples of holo-BLA in the absence of nanospheres and CHAPS, of apo-BLA in the absence of nanospheres and CHAPS, of holo-BLA in the presence of 41 mM CHAPS, and of apo-BLA in the presence of 41 mM CHAPS, which were acquired with 32 scans. All samples contain DSS as a reference.

4.2.4 Data processing and analysis

NMR data were processed and analyzed on Silicon Graphics O2 workstations using XWIN-NMR version 2.1 (Bruker Analytik GmbH, Germany). Free induction decays (FIDs) of 2D TOCSY experiments were multiplied by a quadratic cosine function in both t_1 and t_2 , and zero-filled to 4K in t_2 prior to Fourier transformation. A baseline correction in F_2 was applied to the transformed data. FIDs of 1D NOESY experiments were multiplied by a quadratic cosine function and zero-filled to 4K prior to Fourier transformation. A baseline correction was applied to the transformed data. The ^1H chemical shifts are reported relative to DSS.

4.2.5 Adsorption isotherm for the adsorption of BLA on polystyrene nanospheres

The adsorption isotherm was determined using two separate adsorption experiments according to the depletion method. In a typical adsorption experiment, 4 to 40 μl of an 8.2 mM holo-BLA solution in 20 mM sodium phosphate of pH 7.0 was added to an Eppendorf tube that contained 550 μl of a 123 nM polystyrene nanosphere suspension

in 20 mM sodium phosphate buffer of pH 7.0. The tubes were gently shaken at room temperature. Adsorption isotherms were determined after 30 minutes of adsorption. Different protein concentrations were used to construct an adsorption isotherm. The polystyrene nanospheres were separated from free holo-BLA by ultrafiltration during approximately 10 minutes using Vivaspin ultrafiltration devices with a 100 kD molecular weight cut off (Vivascience, Hannover, Germany). Protein adsorption on the filter was checked and found to be negligible. The filter retained more than 99.8% of the polystyrene nanospheres. The concentration of BLA in solution was determined spectrophotometrically using a molar extinction coefficient of $28,540 \text{ M}^{-1}\text{cm}^{-1}$ for BLA at 280 nm.

4.3 Results

Adsorption of BLA on the surface of polystyrene nanospheres induces partial unfolding of adsorbed BLA, resulting in the formation of a molten globule-like conformation (Engel, et al., 2002). Here, ^1H -NMR spectroscopy is used to study in solution the adsorption of holo- and apo-BLA on suspended polystyrene nanospheres and the subsequent displacement of these protein molecules from the nanospheres using the surfactant CHAPS. The effect displacement has on the conformation of the previously adsorbed holo- and apo-BLA molecules is investigated. In addition, the effect of the presence of nanospheres and of surfactant molecules on the ^1H -NMR spectra of holo- and apo-BLA is studied.

4.3.1 Does the surfactant CHAPS affect the conformational properties of BLA?

Not only protein adsorption, but also the presence of a surfactant can affect the conformation of a protein. Although the 1D ^1H -NMR spectrum of holo-BLA is not significantly affected by the presence of 41 mM CHAPS, the corresponding NMR spectrum of apo-BLA is (Fig. 4-1). The ^1H -NMR spectrum of apo-BLA in the presence of 41 mM CHAPS is typical for that of a denatured protein. 2D ^1H -NMR spectroscopy allows the assignment of the individual backbone amide proton resonances of holo-BLA in the presence and absence of 41 mM CHAPS. The effect of CHAPS on these resonances is shown in Fig. 4-2. Only four amide proton resonances (T4, G19, W104, and NH_ϵ of W60) are significantly, but still minimally shifted (ranging between -0.10 and 0.06 ppm), probably due to specific but weak interactions of CHAPS molecules with BLA. The mean of the differences in chemical shifts between the assigned, non-overlapping, 80 amide proton peaks and the 4 Trp NH_ϵ protons of holo-BLA in the presence and in the absence of 41 mM CHAPS is only 0.002 ppm, with a standard deviation of 0.022 ppm. It can thus be concluded that the 3D structure of holo-BLA is

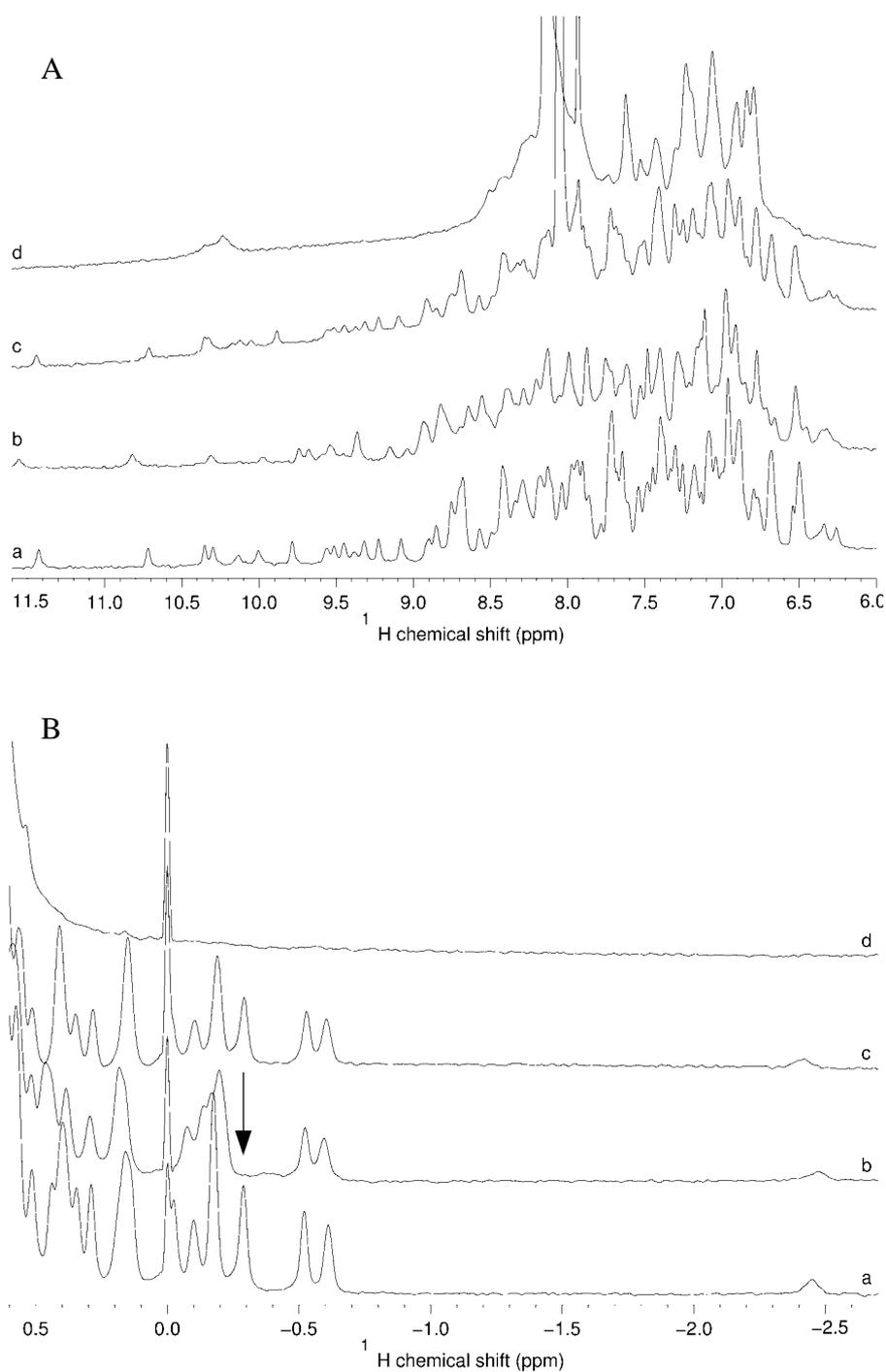


Fig. 4-1. Lowfield amide region (A) and highfield methyl region (B) of the 500 MHz ^1H -NMR spectra of holo-BLA at 35 °C (a), apo-BLA at 20 °C (b), holo-BLA in the presence of 41 mM CHAPS at 35 °C (c), and of apo-BLA in the presence of 41 mM CHAPS at 25 °C (d). All samples contain 10% D_2O and 20 mM sodium phosphate at pH 7.0. The samples of apo-BLA contain 5 mM EDTA. The protein concentration is 0.60 mM. The arrow indicates the resonance position of a δCH_3 group of residue L15 in the ^1H -NMR spectrum of native holo-BLA at -0.3 ppm that is shifted downfield by 0.09 ppm in the ^1H -NMR spectrum of apo-BLA (Wijesinha-Bettoni, et al., 2001). The resonance position of this δCH_3 group in the ^1H -NMR spectrum of holo-BLA is not affected by decreasing the temperature to 20 °C (spectrum not shown).

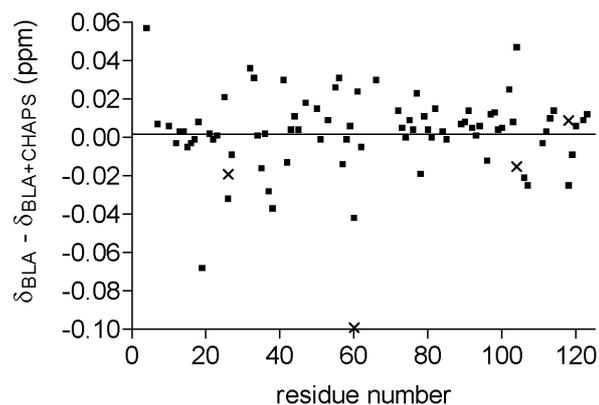


Fig. 4-2. Differences in NH proton chemical shift positions ($\delta_{\text{BLA}} - \delta_{\text{BLA+CHAPS}}$) at 35 °C between native holo-BLA and holo-BLA in the presence of 41 mM CHAPS plotted *versus* amino acid residue number. Chemical shift differences are shown for the assigned, non-overlapping, 80 amide protons (squares) and for the 4 NH_ϵ protons of the tryptophans (crosses) of holo-BLA and are obtained from 500 MHz ^1H -TOCSY spectra. The horizontal line is drawn at the mean of the chemical shift differences observed. Both samples contain 20 mM sodium phosphate buffer at pH 7.0 in 90% H_2O / 10% D_2O .

not significantly affected by the presence of 41 mM CHAPS, whereas this presence causes apo-BLA to unfold.

4.3.2 Adsorption of BLA on suspended polystyrene nanospheres studied by NMR spectroscopy

A 1D ^1H -NMR spectrum of a 7.1% (w/w) colloidal suspension of polystyrene nanospheres in the absence of BLA has been acquired (Fig. 4-3, spectrum a). Although polystyrene contains many protons, the corresponding proton signal cannot be detected in the spectrum shown due to severe line broadening as a result of the slow rotation of the polystyrene nanospheres on the NMR chemical shift time scale. Subsequently, small amounts of a concentrated solution of holo-BLA are added to the NMR tube containing the polystyrene nanospheres, resulting in final BLA concentrations of 0.11, 0.21 and 0.31 mM, respectively. No proton signals are observed in the corresponding ^1H -NMR spectra (Fig. 4-3, spectra b, c, and d). All added holo-BLA molecules adsorb on the surface of the nanospheres, and no free BLA is present in solution. Again, due to the slow rotation of the nanospheres, which are now partially covered by adsorbed protein molecules, no signal is seen in the corresponding ^1H -NMR spectra. Further addition of BLA, resulting in a final BLA concentration of 0.41 mM, leads to signal that corresponds with the 1D ^1H -NMR spectrum of native holo-BLA (Fig. 4-3, spectrum e). Under these conditions, the polystyrene nanospheres are fully covered with a monolayer of adsorbed BLA molecules and the excess BLA molecules do not adsorb on these nanospheres. The latter BLA molecules, which rotate freely in solution, are detected by NMR spectroscopy and cause a ^1H -NMR spectrum typical for native holo-BLA. Thus, free holo-BLA does not unfold in the presence of fully BLA-covered nanospheres, nor

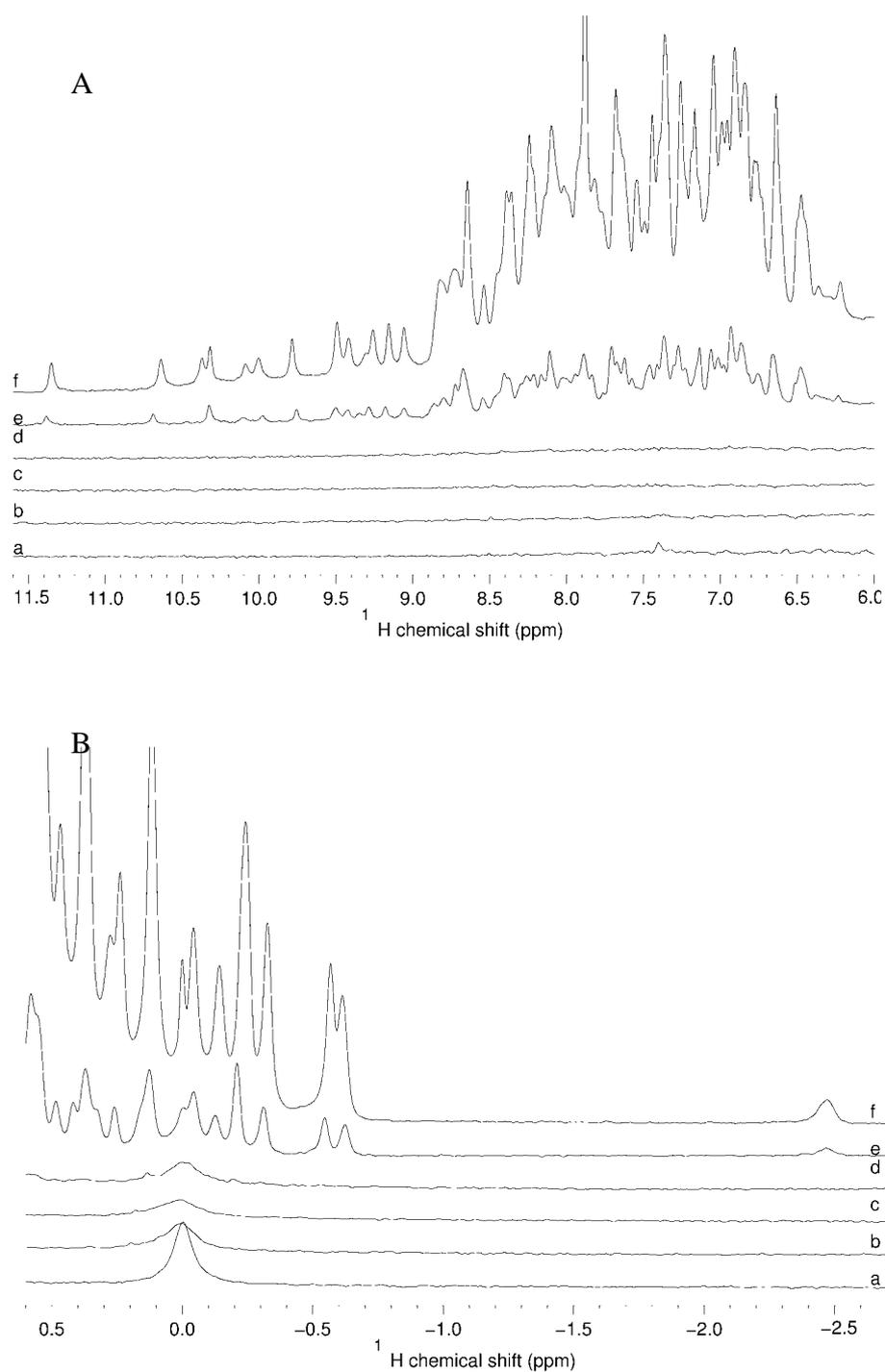


Fig. 4-3. Lowfield amide region (A) and highfield methyl region (B) of the 500 MHz ^1H -NMR spectra of 123 nM polystyrene nanospheres (a), of 123 nM polystyrene nanospheres to which small amounts of an 8 mM holo-BLA solution are added resulting in a final BLA concentration of 0.11 mM (b), 0.21 mM (c), 0.31 mM (d) and 0.41 mM (e), respectively. The ^1H -NMR spectrum of 123 nM polystyrene nanospheres in the presence of 0.41 mM holo-BLA to which subsequently CHAPS is added to a final concentration of 19 mM to displace the adsorbed protein from the nanospheres is shown in (f). The resonance position of DSS at 0 ppm is broadened due to interaction with the nanospheres. All samples contain 20 mM sodium acetate buffer at pH 5.9. The spectra are acquired at 35 °C.

does it lose its calcium ion under these conditions. Clearly, the polystyrene nanospheres do not significantly affect the quality of the obtained $^1\text{H-NMR}$ spectra of free, native holo-BLA. In addition, the presence of polystyrene nanospheres does not affect the chemical shifts of the backbone amide protons of holo-BLA molecules rotating freely in solution, as is shown later.

Apo-BLA is adsorbed on the polystyrene nanospheres in an experiment similar to the one described above for holo-BLA. Addition of a small amount of a concentrated solution of apo-BLA to the nanospheres, resulting in a final BLA concentration of 0.22 mM, leads to adsorption of all added protein, with no free apo-BLA left in solution (Fig. 4-4, spectrum a). Further addition of apo-BLA, resulting in a final BLA concentration of 0.42 mM, leads to monolayer coverage of the nanospheres, and the excess native apo-BLA, which is freely rotating in solution, is seen in the 1D $^1\text{H-NMR}$ spectrum (Fig. 4-4, spectrum b). The apo-form of BLA is easily recognized in a 1D $^1\text{H-NMR}$ spectrum by the absence of the characteristic peak of a δCH_3 group of residue L15 of holo-BLA at -0.3 ppm (Wijesinha-Bettoni, et al., 2001) (compare Fig. 4-4B, spectrum b and Fig. 4-1B, spectrum b). Again, as observed for holo-BLA, the presence of the fully BLA-covered polystyrene nanospheres does not affect the conformation of free apo-BLA.

The presented 1D $^1\text{H-NMR}$ spectra contain information about the amount of adsorbed BLA, which can be calculated from the amount of BLA that is added to the NMR tube and the amount of free BLA that is visible in the corresponding $^1\text{H-NMR}$ spectrum. The latter amount is easily determined by comparing the $^1\text{H-NMR}$ spectrum of free BLA in the presence of fully BLA-covered nanospheres with the one of a reference sample of native BLA with a known protein concentration. Fig. 4-5 shows the thus obtained adsorbed amounts of holo- and apo-BLA. For comparison, Fig. 4-5 also shows the adsorption isotherm for the adsorption of holo-BLA on the polystyrene nanospheres as determined by a separate conventional method, which involves the separation of free BLA from the polystyrene nanospheres by ultrafiltration (see materials and methods section). The steep rise of the adsorption isotherm at low BLA concentrations indicates high affinity adsorption behaviour of holo-BLA. A well-defined plateau value is observed that corresponds to 1.3 mg/m^2 BLA adsorbed as a monolayer on the surface of the polystyrene nanospheres. The data obtained by NMR spectroscopy agree with those obtained by ultrafiltration and show that indeed NMR spectroscopy can be used to determine the amount of adsorbed protein molecules.

The plateau value determined in chapter 2 for the adsorption of holo-BLA on similar polystyrene nanospheres is significantly higher (3 mg/m^2) (Engel, et al., 2002) than the one determined here. The experimental conditions used here, i.e. a nanosphere concentration of 7.1% (w/w) compared to 0.025% (w/w) used in chapter 2, and the absence of a stabilizing agent, probably promote some aggregation of the polystyrene

nanospheres. As a result, the polystyrene nanosphere surface area available for adsorption of holo-BLA is overestimated, leading to the low plateau value observed here.

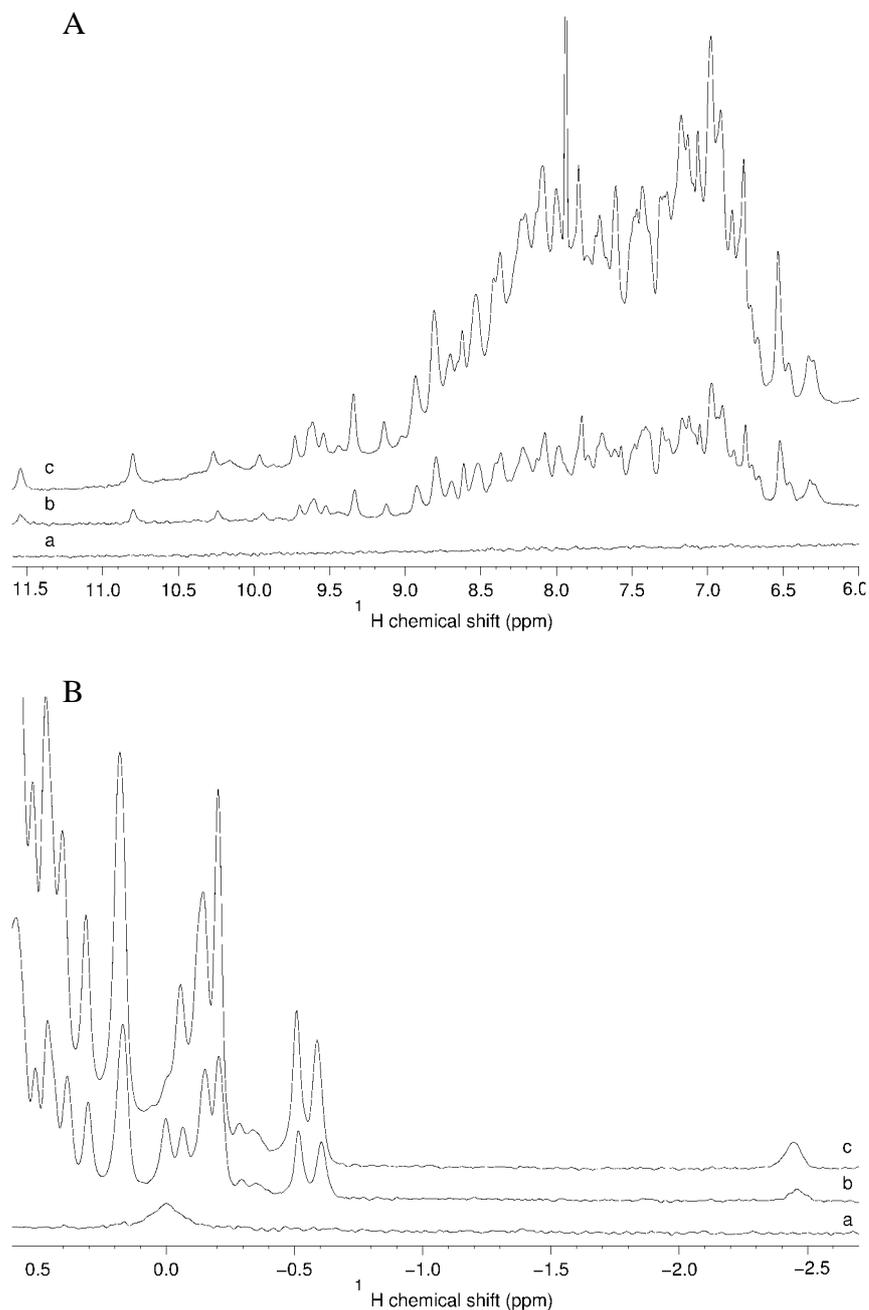


Fig. 4-4. Lowfield amide region (A) and highfield methyl region (B) of the 500 MHz ^1H -NMR spectra of 123 nM polystyrene nanospheres to which small amounts of an 8.0 mM apo-BLA solution are added resulting in a final BLA concentration of 0.22 mM (a), and 0.42 mM (b), respectively. The ^1H -NMR spectrum of 123 nM polystyrene nanospheres in the presence of 0.42 mM apo-BLA to which subsequently CHAPS is added to a final concentration of 10 mM to displace the adsorbed protein from the nanospheres is shown in (c). The resonance position of DSS at 0 ppm is broadened due to interaction with the nanospheres. All samples contain 20 mM sodium phosphate buffer with 0.26 mM EDTA at pH 7.4. The spectra are acquired at 24 $^{\circ}\text{C}$.

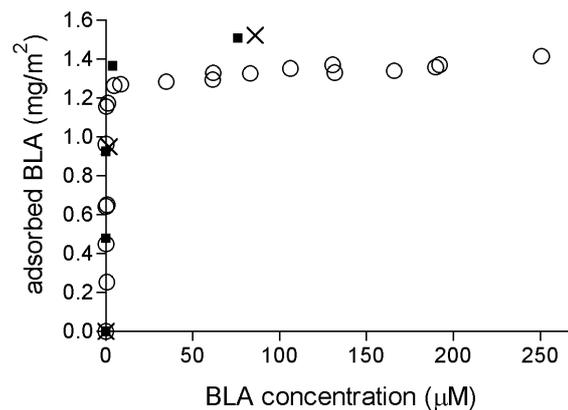


Fig. 4-5. Adsorption isotherm of the adsorption of holo-BLA on polystyrene nanospheres. The adsorption isotherm as determined by ultrafiltration in 20 mM sodium phosphate buffer of pH 7.0, at 20 °C is shown by open circles. The corresponding data obtained by NMR spectroscopy in 20 mM sodium acetate buffer at pH 5.9, at 35 °C are shown by filled squares. The data obtained by NMR spectroscopy for the adsorption of apo-BLA on polystyrene nanospheres in 20 mM sodium phosphate buffer with 0.26 mM EDTA at pH 7.4, at 24 °C are shown by crosses.

4.3.3 Complete refolding of BLA after its displacement from the polystyrene nanospheres by CHAPS

Up to 41 mM CHAPS does not affect the conformation of holo-BLA, as demonstrated (Figs. 4-1 and 4-2). Consequently, CHAPS can be used to study the displacement and subsequent refolding of BLA molecules that are adsorbed in a partially unfolded state on the surface of the polystyrene nanospheres. Addition of CHAPS to a sample of polystyrene nanospheres covered with a monolayer of adsorbed BLA molecules results in the displacement of these adsorbed BLA molecules. This can be seen by the clear increase of the protein signal in the 1D ^1H -NMR spectra acquired after the addition of CHAPS to samples of adsorbed holo-BLA and of adsorbed apo-BLA (Fig. 4-3, compare spectra e and f, and Fig. 4-4, compare spectra b and c, respectively). From these spectra, it is estimated that 70% of the adsorbed holo-BLA is displaced by 19 mM CHAPS and that 47% of the adsorbed apo-BLA is displaced by 10 mM CHAPS. Displaced holo- and apo-BLA give rise to 1D ^1H -NMR spectra characteristic for their native states. Thus, the surfactant CHAPS enables the displacement and subsequent refolding of BLA molecules that were previously adsorbed in a partially unfolded state on the polystyrene nanospheres.

Detailed conformational information at the residue level about the previously adsorbed and subsequently displaced holo-BLA in the presence of the polystyrene nanospheres and CHAPS is obtained from a 2D NMR spectrum of the displaced protein (Fig. 4-6) Despite the presence of 7.1% (w/w) polystyrene nanospheres that are covered with CHAPS and with a fraction of the total amount of BLA, the quality of the 2D TOCSY ^1H -NMR spectrum of displaced holo-BLA is surprisingly good. As a result,

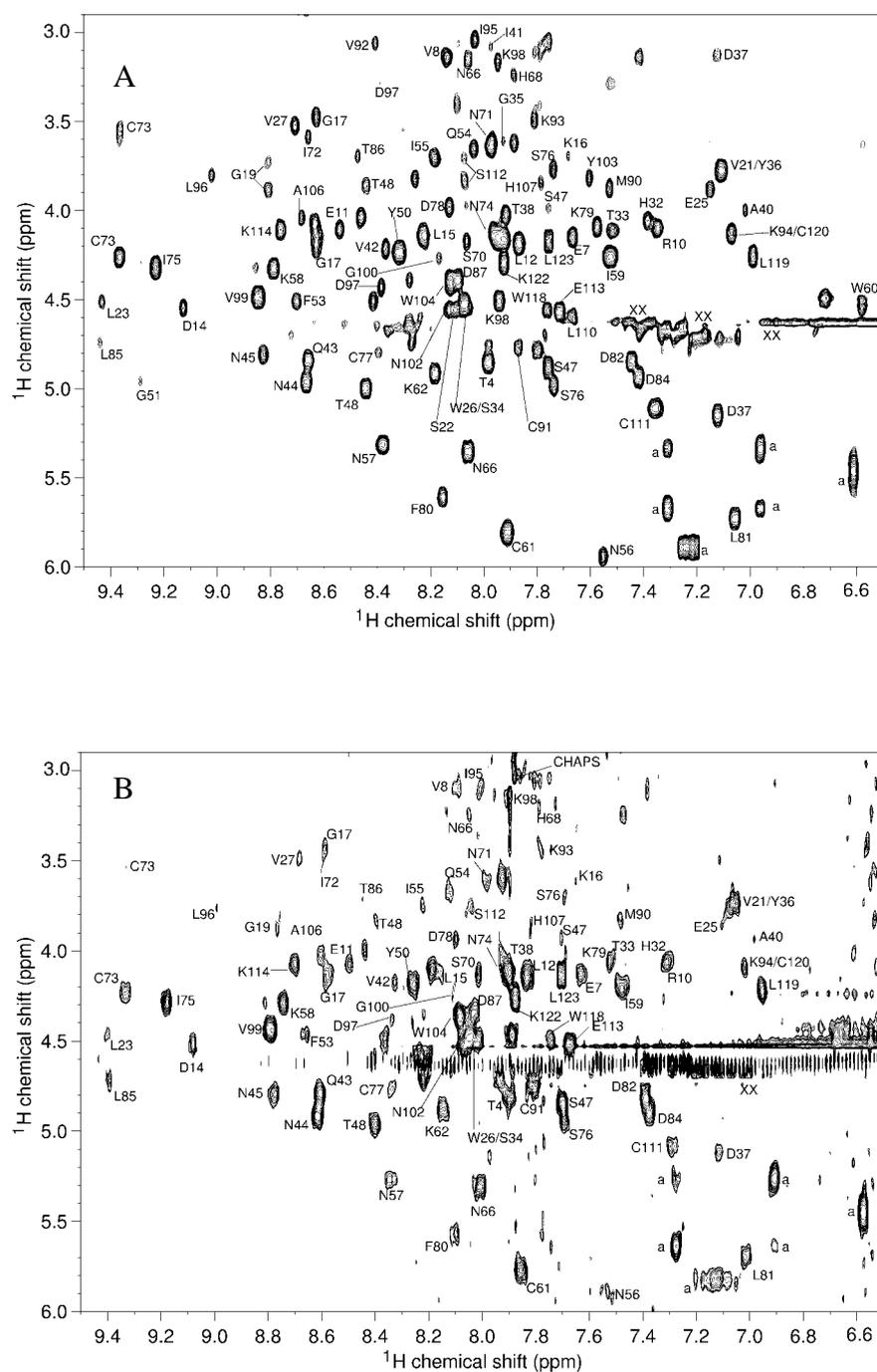


Fig. 4-6. Comparison of part of the 500 MHz ^1H -TOCSY spectra of 0.60 mM holo-BLA in 20 mM sodium phosphate buffer at pH 6.0 (A) and of 0.33 mM displaced holo-BLA in 20 mM sodium acetate buffer at pH 5.9 (B). Displaced holo-BLA is obtained by the addition of CHAPS to a final concentration of 19 mM to the NMR tube containing 123 nM polystyrene nanospheres covered by a monolayer of adsorbed BLA molecules. The spectra are acquired at 35 °C. The crosspeak assignments are indicated with the one-letter amino acid code and corresponding sequence number. Crosspeaks due to aromatic protons are labeled (a). The horizontal residual water signal is labeled (XX). The signal-to-noise ratio of the spectrum of displaced BLA (B) is lower compared to that of native BLA (A) due to a lower protein concentration and a lower number of scans acquired (32 compared to 48). In addition, the receiver gain had to be lowered to 32 instead of 128, due to the signals arising from acetate and CHAPS in the sample of displaced BLA.

most of the assigned cross peaks associated with amide protons in the TOCSY spectrum of native BLA can also be assigned in the TOCSY spectrum of displaced BLA in the presence of CHAPS and nanospheres. The differences in chemical shift positions of the amide protons between native BLA and displaced BLA average around 0.049 ppm, with a standard deviation of 0.018 ppm (includes 71 assigned, non-overlapping, backbone amide protons and 4 Trp NH_ε protons of BLA) (Fig. 4-7). The small 0.049 ppm mean chemical shift difference is probably caused by slight differences in the experimental conditions between the two BLA samples. Displaced BLA is in an acetate buffer at pH 5.9, whereas native BLA is in a phosphate buffer at pH 6.0.

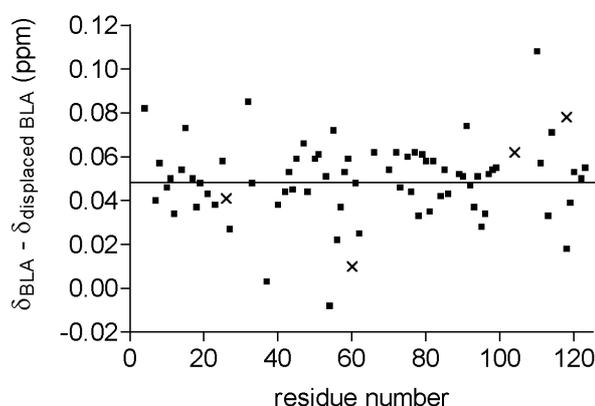


Fig. 4-7. Differences in NH proton chemical shift positions ($\delta_{\text{BLA}} - \delta_{\text{displaced BLA}}$) at 35 °C between native holo-BLA in 20 mM sodium phosphate buffer at pH 6.0 and displaced holo-BLA in the presence of 19 mM CHAPS and 123 nM nanospheres in 20 mM sodium acetate buffer at pH 5.9. Chemical shift differences are shown for the 71 assigned, non-overlapping, backbone amide protons (squares) and for the 4 NH_ε protons of the tryptophans (crosses) and are obtained from the 500 MHz ¹H-TOCSY spectra shown in Fig. 4-6. The horizontal line is drawn at the mean of the chemical shift differences observed.

In summary, the data presented show that displaced BLA has the same conformation as native BLA. The adsorption-induced partial unfolding of BLA is thus completely reversible at the residue-level upon displacement of adsorbed BLA from the polystyrene nanospheres by the use of the surfactant CHAPS.

4.4 Discussion

Here, it is shown for the first time that the adsorption of protein molecules on a solid interface, i.e. polystyrene nanospheres, can be studied in solution by NMR spectroscopy. In spite of the presence of the nanospheres, it is possible to determine the three-dimensional structure of non-adsorbed BLA by NMR spectroscopy. This is feasible because the quality of the corresponding ¹H-NMR spectra is surprisingly good.

1D and 2D ¹H-NMR spectra show that the conformation of holo-BLA that is adsorbed and subsequently displaced from polystyrene nanospheres by the surfactant

CHAPS is identical at the atomic level to that of native holo-BLA. Consequently, the conformational changes of holo-BLA induced by its adsorption on a hydrophobic interface are fully reversible upon displacement of these protein molecules using the surfactant CHAPS.

The efficient displacement by CHAPS and the subsequent refolding of the previously adsorbed BLA molecules are the now fulfilled essential requirements that enable the indirect study, at the residue level, of the orientation, conformation and stability of BLA adsorbed on polystyrene nanospheres by hydrogen/deuterium (H/D) exchange and 2D ^1H -NMR spectroscopy (chapter 5). As the displaced BLA molecules refold to the native state, they have many amide protons that become protected against exchange with the deuterated solvent. This ensures that sufficient time is available to make an NMR sample of the displaced protein and to subsequently acquire 2D ^1H -NMR spectra without substantial further H/D exchange occurring. The ^1H -NMR resonance positions of displaced BLA are identical to those of native BLA, thus it is straightforward to detect the H/D exchange of the individual amide protons of adsorbed BLA. This exchange behaviour informs about the detailed conformational properties of adsorbed BLA.

In contrast to methods based on the physical separation of proteins from suspended solid particles, NMR spectroscopy allows the non-invasive estimation of the amount of proteins adsorbed on suspended nanospheres. The severe broadening of the protein resonances due to the long rotational correlation time of the adsorbed protein molecules is favourably exploited in this method. The amount of protein molecules and surfactant molecules that are free in solution can be determined by NMR spectroscopy without disturbing the equilibrium of the system. The absence of protein signal in the ^1H -NMR spectra obtained from polystyrene nanospheres in the presence of low amounts of protein indicates that BLA adsorbs to these nanospheres with high affinity. This is confirmed by the steep slope of the initial part of the adsorption isotherm for BLA adsorption on polystyrene nanospheres as determined by ultrafiltration. The adsorption isotherm values as determined by NMR spectroscopy differ slightly from those obtained by ultrafiltration (Fig. 4-5). This can be explained by the undesired non-equilibrium situation in the case of the ultrafiltration experiments, which is caused by the increase of the adsorbed protein concentration relative to the concentration of free protein during the course of the ultrafiltration experiment. Hence, the adsorbed amount of BLA on polystyrene nanospheres as determined by ultrafiltration is expected to be lower compared to the amount determined by NMR spectroscopy, as is indeed observed (Fig. 4-5).

Not only do the 1D ^1H -NMR spectra inform about the amount of adsorbed BLA, the amount of adsorbed CHAPS can also be determined from these spectra. This amount is

calculated by subtracting the amount of CHAPS that is freely rotating in solution, as inferred from the CHAPS signal in the $^1\text{H-NMR}$ spectrum of displaced BLA in the presence of CHAPS and nanospheres, from the total amount of CHAPS added to the solution. The amount of free CHAPS is determined via the comparison of its $^1\text{H-NMR}$ spectrum with the one of a reference spectrum of CHAPS with a known concentration. The amount of free CHAPS in the sample of displaced holo-BLA thus determined is approximately 1 mM, which is only 5% of the total amount of CHAPS added. The amount of free CHAPS in solution after displacement of apo-BLA from the polystyrene nanospheres thus determined is approximately 0.7 mM, which is only 7% of the total amount of CHAPS added. This shows that the majority (93 – 95%) of the added CHAPS molecules thus adsorb on the surface of the polystyrene nanospheres. This high affinity of CHAPS for the polystyrene nanospheres causes the efficient displacement of the adsorbed BLA molecules. More than 90% of the adsorbed BLA molecules can be displaced at high CHAPS concentrations (Engel, et al., 2003).

The observation by NMR spectroscopy that only a few percent of the total amount of added CHAPS molecules is present freely in solution after displacement of the adsorbed protein explains why displaced apo-BLA does not unfold under these conditions. The amount of free CHAPS present in the sample of displaced apo-BLA is only 0.7 mM, which is insufficient to unfold the protein, whereas 41 mM of CHAPS causes denaturation of apo-BLA (Fig. 4-1, spectrum d). CHAPS does not significantly affect the chemical shifts of the backbone amide protons of displaced holo- and apo-BLA, as shown by NMR spectroscopy. The results presented here show that CHAPS, at low concentrations, indeed is a non-denaturing surfactant (Hjelmeland, 1990). Hence, CHAPS is very suitable for the displacement of previously adsorbed proteins from a hydrophobic interface. This allows the indirect study of the structural properties of adsorbed proteins by NMR spectroscopy according to the procedures described above. CHAPS can even be used in combination with proteins with a low thermodynamic stability, like apo-BLA, as it does not unfold these proteins at low surfactant concentrations.

Since both holo- and apo-BLA are shown here to adsorb on polystyrene nanospheres, the calcium ion present in holo-BLA is not needed for adsorption of BLA on the interface. This supports the former observations that holo-BLA loses its calcium ion upon adsorption on polystyrene nanospheres (Engel, et al., 2002; Galisteo, et al., 1995a; b).

The protein adsorption study by NMR spectroscopy presented here is focused on one protein (BLA) and on one surface (suspended polystyrene nanospheres), but should be applicable to the adsorption of other proteins on other surfaces as well. Currently, the chosen protein molecules need to be smaller than approximately 40 kD to allow the

assignment of their backbone amide proton resonances in the corresponding solution NMR spectra. Assignment of the protein NMR spectrum is not needed for the determination of the amounts of adsorbed protein by NMR spectroscopy. The chosen solid adsorbents need to form a stable suspension in water and should provide sufficient surface area for a substantial amount of protein molecules to be adsorbed. The method described here for the use of NMR spectroscopy to study proteins adsorbed on nanospheres could even be used to study protein adsorption on liquid interfaces, like the oil/water interface in an emulsion.

5 A protein folding intermediate trapped by adsorption

Adsorption-induced conformational changes of proteins play an essential role during protein adsorption on interfaces. Unfortunately, due to considerable experimental difficulties, detailed information at the molecular level about these conformational changes is lacking. This hampers the further development of the theory of protein adsorption. Here, hydrogen/deuterium (H/D) exchange and two-dimensional solution-state NMR spectroscopy are used to study protein adsorption in detail. The orientation, conformation, and local stability at the residue level of bovine α -lactalbumin (BLA) adsorbed on polystyrene nanospheres are characterized. It is shown that the majority ($\pm 60\%$) of the adsorbed protein molecules has conformational properties similar to BLA molecules in the acid-denatured state (A-state). A folding intermediate of BLA is thus induced and trapped by adsorption of the protein on the hydrophobic interface. Several residues, clustered on one side of the adsorbed folding intermediate of BLA, have altered amide proton exchange protection factors compared to those of the A-state of BLA. This side preferentially interacts with the interface, and includes residues in helix C, the calcium binding site and part of the β -domain. Local unfolding of this interacting part of the adsorbed protein is proposed to initiate the adsorption-induced unfolding of BLA. Adsorption-induced protein unfolding apparently resembles more the mechanical unfolding of a protein than the global unfolding of a protein as induced by denaturant, pH or pressure. The minority ($\pm 40\%$) of adsorbed BLA molecules has significantly lower amide proton exchange rates than the adsorbed BLA folding intermediate has. These BLA molecules arrive at a later stage during the adsorption process and cannot adapt to the interface by unfolding to the A-state because two-dimensional macromolecular crowding on the nanosphere surface prevents them to. Near-UV CD spectroscopy confirms the presence of the two structurally different populations of adsorbed BLA molecules in a ratio similar to the one found by NMR spectroscopy. This study not only provides new insights into the mechanisms of adsorption-induced protein unfolding, the method presented is also valuable for the study of folding intermediates of other proteins. Adsorption of proteins on hydrophobic interfaces can be used as a valuable method to trap and subsequently study otherwise marginally stable protein folding intermediates.

5.1 Introduction

Adsorption of protein molecules on interfaces plays a fundamental role in a large number of biological and man-made systems. Protein adsorption occurs in the presence of membranes and medical implants, and plays an important role in, among others, chromatography and nanotechnology. It is now well understood that adsorption-induced conformational changes are an integral part of the adsorption process, and thus they cannot be ignored in the current theory of protein adsorption (Haynes, et al., 1995). A few studies suggest that protein folding intermediates possibly play a role in protein adsorption (Billsten, et al., 1997; Dickinson, et al., 1994; Engel, et al., 2002). Unfortunately, detailed information at the sub-molecular level about the conformational changes that are induced by protein adsorption is lacking. Here, the orientation, conformation and local stability of bovine α -lactalbumin (BLA) adsorbed on polystyrene nanospheres is characterized at the residue level by hydrogen/deuterium (H/D) exchange and two-dimensional Nuclear Magnetic Resonance (NMR) spectroscopy.

Direct detection of the conformational properties of adsorbed proteins by solution-state NMR spectroscopy is very difficult due to the extreme line broadening of the corresponding NMR signal. However, indirect information can be extracted by allowing amide hydrogen atoms of adsorbed protein molecules to exchange with deuterated solvent during a defined period. After quenching of the exchange and displacement of the adsorbed protein molecules from the surface, this exchange can be quantified in solution by two-dimensional ^1H -NMR spectroscopy. In chapter 3, a method is developed to obtain native displaced protein after its adsorption, using a non-denaturing surfactant (Engel, et al., 2003).

In the work reported here, BLA is chosen to study protein adsorption because the structure, folding and the stability of the native state and of intermediate states of this protein have been thoroughly investigated. In addition, the interaction of BLA with a variety of interfaces has been studied with several techniques (Engel, et al., 2002; Engel, et al., 2003; Halskau, et al., 2002; Haynes, et al., 1995). Polystyrene nanospheres were chosen as adsorbent because they were successfully used to study the adsorption of BLA in previous studies (Engel, et al., 2002; Engel, et al., 2003).

In the few reported studies of the conformation of adsorbed proteins using H/D exchange and NMR spectroscopy, only limited information about the conformation and the local stability of the adsorbed protein (i.e. lysozyme) could be obtained. The mentioned studies report exchange rates of only 4 lysozyme residues (Nagadome, et al., 1993) or exchange information that is based on just a single point in time (Keire, et al., 1992; McNay, et al., 1999). Mass spectrometry was used with the intention to study

H/D exchange of adsorbed myoglobin, but only the exchange behaviour of large peptide fragments could be characterized (Buijs, et al., 2003). In contrast to the limited information obtained in the former studies, here many amide proton exchange rates of an adsorbed protein are reported. Protection factors of 28 individual amide hydrogens of BLA adsorbed on a hydrophobic interface could be determined. The amide proton exchange behaviour of adsorbed BLA molecules is similar to the exchange behaviour of the corresponding protons of the acid-induced molten globule state (A-state) of BLA. A bovine α -lactalbumin folding intermediate is induced and trapped by adsorption of BLA on a hydrophobic interface.

5.2 Materials and methods

5.2.1 Materials

Bovine α -lactalbumin (L-5385), with approximately 2 moles calcium ions per mole BLA (see chapter 3), and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS, C5070) were obtained from Sigma. Anhydrous CaCl_2 was obtained from Janssen Chimica. Polystyrene nanospheres with a radius of 60 ± 1 nm (Interfacial Dynamics Corporation, Portland, USA) and with a radius of 23 ± 1 nm (Polymer Laboratories, Heerlen, The Netherlands) were supplied as a concentrated colloidal suspension in water. Nanosphere suspensions were diluted with buffer before use. The pH was measured with a standard glass-electrode and the pH readings in $^2\text{H}_2\text{O}$ (pD) are uncorrected for isotope effects. A buffer containing 20 mM sodium acetate (Merck, Germany) at pD 5.5 was used in all experiments, unless mentioned otherwise. Nanopure water (Sybron Barnstead NANOpure II) or deuterium oxide (99.9%, Cambridge Isotope Laboratories, Inc., USA) was used as a solvent for the preparation of the buffers.

5.2.2 NMR spectroscopy and sample preparation

All NMR experiments were recorded at 35 °C on a Bruker AMX500 equipped with a triple resonance 5 mm inverse probe. Samples were prepared from a filtered (0.2 μm , Schleicher & Schuell GmbH, Germany) stock solution of 8.0 to 8.5 mM BLA in a sodium acetate buffer of pH 5.7. The sample used for resonance assignments contained 0.60 mM BLA in 19 mM sodium phosphate at pH 7.0 in 90% H_2O /10% $^2\text{H}_2\text{O}$. The concentration of BLA was measured spectrophotometrically using a molar extinction coefficient of $28,540 \text{ M}^{-1}\text{cm}^{-1}$ for BLA at 280 nm. Cross peaks were assigned using total correlation spectroscopy (TOCSY), double quantum filtered correlation spectroscopy (DQF-COSY) and nuclear Overhauser effect spectroscopy (NOESY) two-dimensional ^1H -NMR spectra at 35 °C and using reported assignments (Forge, et al., 1999). The

TOCSY (H/D exchange) spectra were acquired using a mixing time of 40 ms with 2048 complex points in t_2 (number of scans 8) and with 350 complex points in t_1 . The spectral widths were 8064 Hz in both t_2 and t_1 . All samples contained 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) as a reference.

5.2.3 NMR Data processing and analysis

NMR data were processed and analyzed on Silicon Graphics O2 workstations using XWIN-NMR version 2.1 (Bruker Analytik GmbH, Germany). Free induction decays (FIDs) were multiplied by a quadratic cosine function in both t_1 and t_2 , and zero-filled to 2K in t_2 prior to Fourier transformation. A baseline correction in F_2 was applied to the transformed data. The ^1H chemical shifts are reported relative to DSS.

5.2.4 H/D exchange of adsorbed BLA

In a typical exchange experiment, a 50 ml polypropylene tube (Greiner Bio-One Inc., USA) containing a magnetic stirring bar and 21.50 ml of 9.1 nM polystyrene nanospheres (radius 60 nm) in 20 mM sodium acetate buffer in $^2\text{H}_2\text{O}$ at pD 5.5 was incubated for at least 20 minutes in a thermostatic water bath at 20 °C. The total surface area of the nanospheres is approximately 5 m². Exchange was initiated by pipeting 45 μl of 8.3 mM BLA in 20 mM sodium acetate in H_2O at pH 5.7 in the stirred nanosphere suspension. Adsorption of BLA on the nanospheres is fast (within milliseconds)(Engel, et al., 2002) compared to the time scale on which H/D exchange occurs (minutes). Separate experiments showed that all BLA molecules adsorb on the nanospheres under these conditions, and no free BLA could be detected in solution. After a certain period, ranging from 5 seconds to 8 minutes, H/D exchange was quenched by adding 1.38 ml of 5 M CaCl_2 in $^2\text{H}_2\text{O}$, and by the subsequent immediate addition of 112 μl of 0.64 M CHAPS in $^2\text{H}_2\text{O}$. This results in a pD of 5.2, a final CHAPS concentration of 3.1 mM, a final CaCl_2 concentration of 300 mM, and a final H_2O content of approximately 6%. The presence of 3.1 mM CHAPS results in the immediate irreversible displacement of more than 90% of the adsorbed BLA molecules and in the subsequent refolding of these displaced molecules (Engel, et al., 2003). Refolding of the BLA molecules induced by displacement from the nanospheres significantly quenches H/D exchange for many displaced BLA backbone amide protons compared to their exchange in the partially unfolded adsorbed state of BLA. Additional quenching is obtained by the decrease of the pD from 5.5 to 5.2 and by the increase of the CaCl_2 concentration from 30 μM to 300 mM. The displaced BLA molecules were separated from the polystyrene nanospheres using an ultrafiltration filter with a 100 kD molecular weight cut off (Centricon Plus-80, Millipore Corporation, USA) at 5 °C during 20 to 30 minutes. Next, the filtrate was concentrated to a protein concentration of approximately 0.3 mM using

an ultrafiltration filter with a 5 kD molecular weight cut off (Centricon Plus-80, Millipore Corporation, USA) at 5 °C during 8 to 10 minutes. DSS was added to the sample just before measuring the TOCSY spectrum. Protein adsorption on the 100 kD ultrafiltration filter was checked and found to be negligible. The 100 kD filter retained more than 99.8% of the polystyrene nanospheres. The remaining 0.2% of nanospheres present in the samples did not affect the NMR experiments. Reference experiments were performed under identical conditions, except that the displacement agent CHAPS was now added to the nanosphere suspension before the addition of BLA. This ensures identical experimental conditions (pH, time of H/D exchange, presence of particles, concentration of reagents) but without protein adsorption taking place. The period between quenching of H/D exchange, which includes protein displacement, and the acquisition of the two-dimensional TOCSY spectra ranged from 90 to 140 minutes.

5.2.5 Hydrogen exchange data analysis

Maximum peak intensities were measured of each observed NH-C_αH cross peak, or of the corresponding diagonal peak (where required) in a TOCSY spectrum using XWIN-NMR. The relative peak intensity was obtained by dividing the maximum intensity of each peak by the average of the intensities of five cross peaks resulting from non-exchangeable aromatic protons. The relative time-dependent peak intensities were fitted to a single exponential decay function (Equation 5-1) using the Levenberg-Marquardt non-linear regression method of the computer program GraphPad Prism (version 3.03 GraphPad Software Inc., USA).

$$I(t) = I(\infty) + C * \exp(-k_{ex} * t) \quad (\text{Eq. 5-1})$$

In Equation 5-1, t is the time between initiation of the H/D exchange and quenching of this exchange, $I(\infty)$ is the peak intensity at infinite time, C is the pre-exponential factor, and k_{ex} is the amide proton exchange rate. The computer program derives standard errors for the best-fit values of each of the mentioned variables. These standard errors are used for the calculation of the standard errors in the data reported in Figs. 5-3 and 5-5. Protection factors were calculated by dividing the intrinsic amide proton exchange rates (k_{int}) by the measured amide proton exchange rates. The intrinsic amide proton exchange rates were determined using free peptide exchange rates, which were corrected for the effects of the local amino acid sequence and calibrated for the pH and the temperature of the exchange experiment as described by Bai et al., (Bai, et al., 1993) and using the online program 'Sphere' (<http://www.fccc.edu/research/labs/roder/sphere/sphere.html>).

5.2.6 Circular Dichroism spectroscopy

Near-UV circular dichroism (CD) measurements were performed on a Jasco J-715 spectropolarimeter, equipped with a Peltier temperature control system set at 20 °C. Calibration was performed with a solution of ammonium d-10-camphorsulfonate in nanopure water of which the concentration (0.060% (w/v)) was checked spectrophotometrically. Protein concentrations used were 4.5 to 22 μM in a 1 cm quartz cuvette. The samples of native BLA and of adsorbed BLA contained 10 mM Tris/HCl at pH 7.5 and 1 mM CaCl_2 . A sample of BLA in the A-state was made by titrating a solution of BLA in nanopure water with HCl to a pH of 2.1. Eight scans were averaged for the samples of native BLA and of BLA in the A-state. The response time was 1 second; the spectral band width was 1.0 nm. Before analysis of the spectra, a CD spectrum of a blank, which contained all components except BLA was subtracted. The sample of adsorbed BLA contained 4.5 μM BLA and 10 nM polystyrene nanospheres (radius 23 nm), resulting in the same BLA coverage of the nanospheres as compared to the samples used for the H/D exchange experiments. The use of polystyrene nanospheres with a radius of 23 nm instead of 60 nm was required to significantly reduce light scattering and light absorption in the CD measurements. In order to avoid disturbances due to instrumental drift, the sample and blank CD spectra of adsorbed BLA were recorded alternately in sets of 32 scans each. Acquisition of 32 scans of the sample of adsorbed BLA was followed by acquisition of 32 scans of the blank sample. This was repeated 4 times, resulting in 128 scans for each sample. For each set of 32 scans, a fresh sample of BLA adsorbed on the nanospheres was used.

5.3 Results

5.3.1 Determination of the H/D exchange behaviour of adsorbed BLA

In this study, BLA molecules in the native state are added to polystyrene nanospheres in D_2O . Subsequently, all protein molecules spontaneously and rapidly (within the dead time of a stopped-flow fluorescence anisotropy experiment (Engel, et al., 2002)) adsorb on the nanosphere surface, which results in a monolayer of adsorbed BLA molecules. Under these conditions, no free BLA molecules are present in solution. The interface induces partial unfolding of the adsorbed protein molecules with an unfolding rate of 74 s^{-1} , as determined by stopped-flow fluorescence spectroscopy (Engel, et al., 2002). Amide protons of the adsorbed, partially unfolded molecules are allowed to exchange with deuterated solvent during a certain period, which is followed by protein displacement from the nanospheres by the addition of a non-denaturing surfactant under quenched exchange conditions. Under these conditions, displaced BLA molecules rapidly refold to their native state in solution. The latter is concluded from

the corresponding TOCSY spectra since they show indistinguishable proton chemical shifts compared to the TOCSY spectrum of native, non-displaced, BLA in solution (chapter 4).

The H/D exchange behaviour of adsorbed BLA is studied by recording TOCSY spectra of displaced BLA molecules that were previously adsorbed on polystyrene nanospheres during H/D exchange periods ranging from 5 seconds to 8 minutes. These TOCSY spectra show that the majority of the observable amide protons of adsorbed BLA exchange faster than those of native, non-adsorbed BLA in the reference sample (Fig. 5-1). The time-dependent amide proton exchange curves and corresponding exchange rates of 28 residues of adsorbed BLA could be determined. Examples of three such H/D exchange curves are shown in Fig. 5-2. The backbone amide protons of residues D88 and L52 show significant exchange within 8 minutes, whereas the backbone amide proton of L96 does not exchange at all within this period. No detectable amide proton exchange is observed during an 8-minute exchange period for the 28 corresponding residues of native BLA in the reference sample (Fig. 5-2).

5.3.2 Orientation of adsorbed BLA and its resemblance to the A-state of BLA

CD and fluorescence spectroscopy show that BLA adsorbed on polystyrene nanospheres has molten globule properties, i.e. it has preserved secondary structure and disordered tertiary structure (Engel, et al., 2002). The protection factors determined here for the backbone amide protons of 28 residues of adsorbed BLA are of the same order of magnitude as those of the corresponding 28 residues of the acid-induced state (A-state) of BLA (Fig. 5-3). Nine residues of adsorbed BLA (V8, L12, Y50, F53, K58, K62, F80, L81, V99) have, within a 95% confidence interval, protection factors identical to those of the corresponding residues of the A-state of BLA. The protection factors of adsorbed BLA are orders of magnitude lower than those of the corresponding residues of native BLA, the protection factors of which are all higher than 3000 (Forge, et al., 1999). The distribution of protection factors within adsorbed BLA has a similar pattern as the one found for the A-state of BLA. These observations show that the conformational characteristics of these adsorbed BLA molecules are similar, on the residue level, to those of the A-state of BLA, i.e. a folding intermediate of BLA is adsorbed on the polystyrene nanospheres.

Although adsorbed BLA and the A-state of BLA have approximately similar H/D exchange features, differences in the exchange behaviour (considering a 95% confidence interval) are also observed between both states. Eight residues of adsorbed BLA (R10, L52, C73, C77, K79, D82, D88 and L96) have a significantly higher protection factor than the corresponding ones in the A-state. Six residues of adsorbed BLA (I55, C61, I75, C91, I95, K98) have a significantly lower protection factor than the

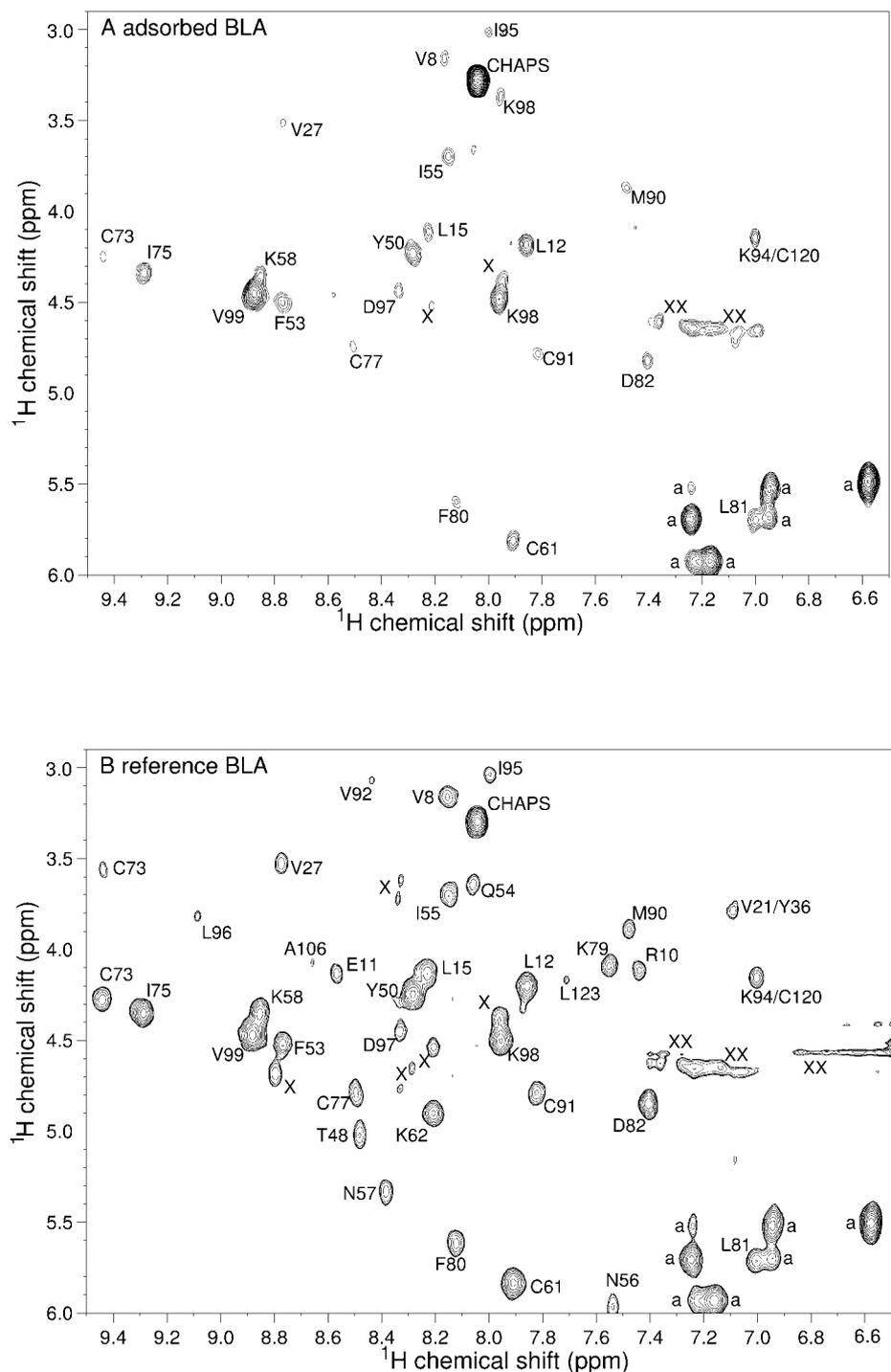


Fig. 5-1. 500 MHz ^1H -TOCSY spectra of displaced BLA the amide protons of which were allowed to exchange with deuterons for a period of 3 minutes at pD 5.5 and 20 °C while BLA was adsorbed on polystyrene nanospheres (A) and of a reference sample of BLA prepared under identical H/D exchange conditions except that adsorption is avoided by the addition of CHAPS to the nanospheres before the addition of BLA (B). The cross peak assignments are indicated with the one-letter amino acid code and corresponding sequence number. Cross peaks from non-exchangeable aromatic protons are labelled (a), unassigned cross peaks are labelled (X) and peaks due to the baseline distortions caused by residual water are labelled (XX). The spectra are acquired in D_2O at 35 °C and pD 5.2. The protein concentration is approximately 0.3 mM and the calcium concentration is approximately 300 mM.

corresponding ones in the A-state. Remarkably, these 14 residues (except R10) are clustered on one side of the BLA molecule (Fig. 5-4). This cluster of residues indicates a region of the adsorbed BLA folding intermediate that has significantly different amide proton exchange behaviour compared to the A-state of BLA, which is most likely due to the interaction of the folding intermediate with the interface.

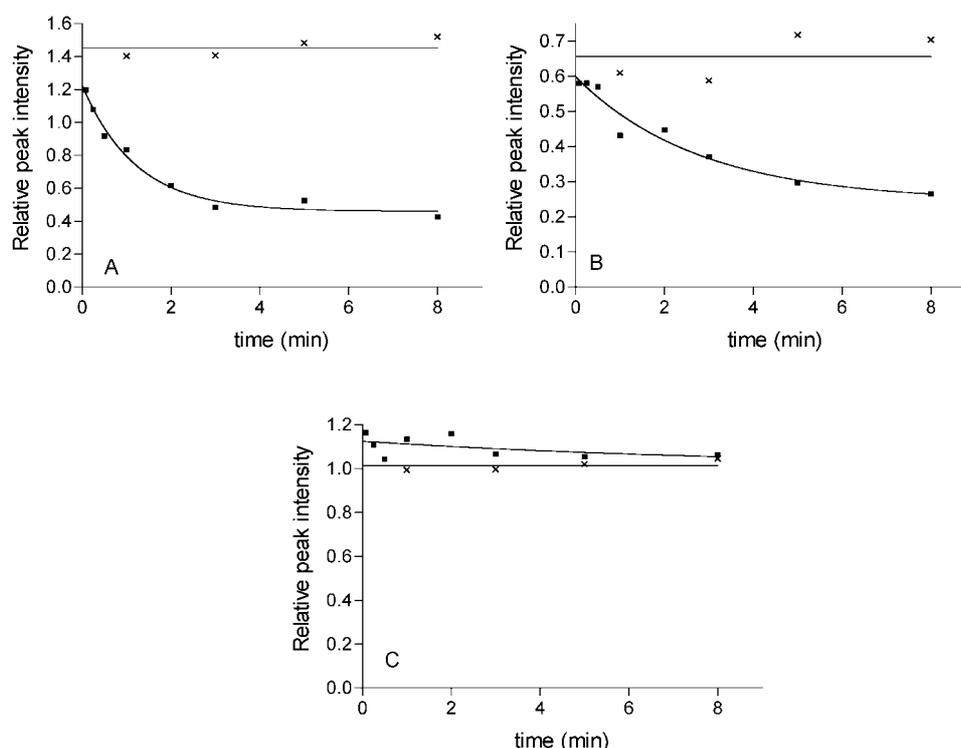


Fig. 5-2. Time-dependent decrease of the backbone amide proton NMR signal of residues D88 (A), L52 (B) and of L96 (C) of BLA adsorbed on polystyrene nanospheres at pD 5.5 and 20 °C as detected after displacement of the protein by CHAPS (squares) and of a reference sample of BLA prepared under identical H/D exchange conditions except that adsorption is avoided by the addition of CHAPS to the nanospheres before the addition of BLA (crosses). The corresponding ^1H -TOCSY spectra are acquired in D_2O at 35 °C and pD 5.2, with a protein concentration of approximately 0.3 mM and a calcium concentration of approximately 300 mM.

Three of the 8 residues that have a significantly higher protection factor in adsorbed BLA compared to the A-state (K79, D82 and D88) are directly involved in ligating the calcium ion in native holo-BLA. As these residues are in a position close to the nanosphere surface, it is likely that the calcium-binding site in adsorbed BLA is distorted, which would result in a drastic reduction of the calcium-binding affinity. Indeed, the calcium ion appears to be absent in BLA adsorbed on the polystyrene nanospheres (Engel, et al., 2002). In adsorbed BLA, the backbone amide protons of the cysteine residues that form two disulfide bonds, i.e. 61-77 and 73-91, show significantly different protection factors compared to those of BLA in the A-state. C61 and C91 have

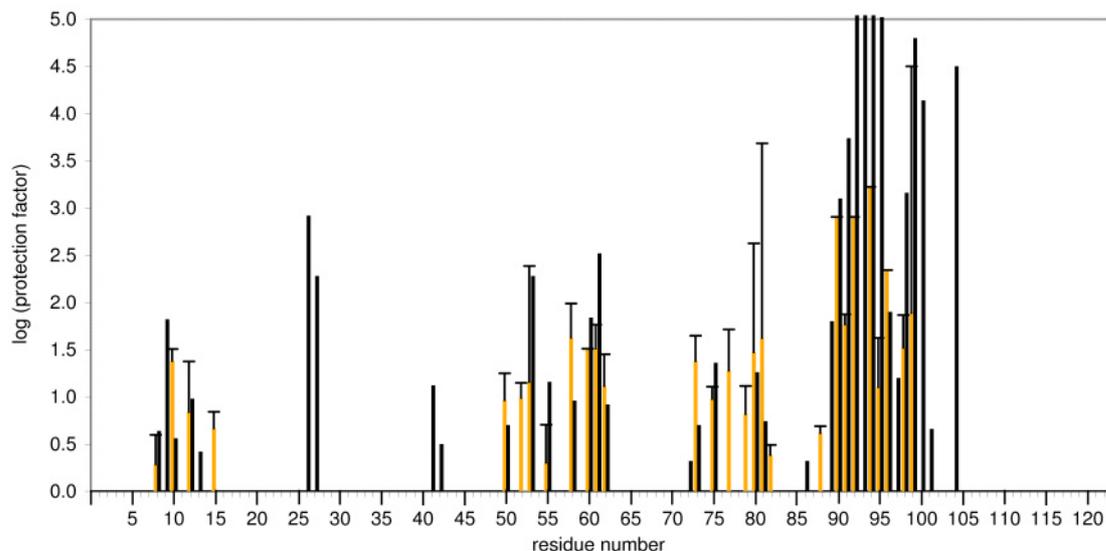


Fig. 5-3. Histograms showing the distribution of amide proton exchange protection factors of adsorbed BLA at pD 5.5 and 20 °C (orange bars) and of the A-state of BLA as determined by Forge et al (Forge, et al., 1999) (black bars). The backbone amide proton exchange of residues 60, 90, 92, 94 and 96 of adsorbed BLA is so slow that an exchange rate could not be obtained. Instead, the upper limit of the corresponding exchange rates is calculated from Eq. 5-1, taking into account a 5% decrease of the amide proton signal intensity during 8 minutes of H/D exchange, and assuming that $I(\infty)$ equals zero: $I(t = 8 \text{ minutes}) = 0.95 * I(t = 0 \text{ minutes})$. The corresponding lower limit of the protection factors of these 5 slow exchanging residues is shown here. Their error bars are set to zero. The backbone amide proton exchange of residues 11, 26, 27, 41, 42, 48, 51, 54, 56, 72, 86, 89, 93, 97, 100, 101 and 104 of adsorbed BLA could not be determined because the corresponding resonances could not be assigned, due to spectral overlap or due to the low signal intensity of the corresponding cross peaks. Residues 52, 77, 79, 82 and 88 are not protected against amide proton H/D exchange in the A-state in solution (Forge, et al., 1999). The error bars show the standard errors of the protection factors of adsorbed BLA as calculated from the standard errors of k_{ex} . Only the positive error bars are shown to improve the readability of the figure.

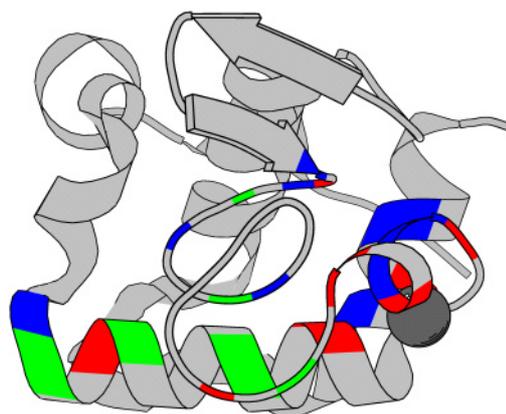


Fig. 5-4. Molscript (Kraulis, 1991) cartoon representation of the X-ray structure of holo-BLA (PDB file: 1F6S)(Chrysina, et al., 2000) in which residues that have identical (blue), or significantly (considering a 95% confidence interval) higher (red) or lower (green), backbone amide proton exchange protection factors of adsorbed BLA compared to the A-state of BLA are shown. The protection factors of the backbone amide protons of five residues of adsorbed BLA (L15, W60, M90, V92, K94) could not be compared to those of the A-state because in case of W60, M90, V92 and K94 only lower limits for the corresponding protection factors of adsorbed BLA could be determined and in case of L15 spectral overlap hindered determination of its exchange rate in the A-state of BLA (Forge, et al., 1999). The grey ball represents the position of the calcium ion in holo-BLA.

a significantly lower and C77 and C73 have a significantly higher protection factor in adsorbed BLA compared to the A-state. The location of these two disulfide bonds is such that they presumably interact with the interface. This is consistent with the time-dependent disulfide shuffling observed in a fraction of the displaced BLA molecules, which is possibly initiated by a few reactive groups on the nanospheres surface, and which is only observed during prolonged homomolecular exchange of adsorbed and subsequently displaced BLA molecules at a surfactant concentration of about 0.3 mM (Engel, et al., 2003).

The indirect detection of the conformation and stability of proteins at interfaces can be successfully done by the H/D exchange method, as shown here. However, there are some limitations to this method. Only those residues of an adsorbed protein can be probed that are also protected against H/D exchange after displacement during the NMR sample preparation time of approximately 2 hours. Hence, it cannot be excluded that additional residues of adsorbed BLA compared to the ones reported here, also have a different amide proton exchange protection relative to the A-state of BLA.

5.3.3 *A minor population of adsorbed BLA molecules has native-like properties*

The relative peak intensities of the exchanging amide protons of adsorbed BLA do not reach zero intensity after 8 minutes of exchange, but apparently reach a plateau value with intensity $I(\infty)$ (as can be seen for residues D88 and L52 in Fig. 5-2). The value of $I(\infty)$ differs for each residue, but can be normalized with respect to the initial peak intensity of each residue, $I(0)$, as obtained from the non-adsorbed BLA reference exchange data. This normalized value could be determined for 19 residues and averages around 0.40 with a standard deviation of 0.13 (Fig. 5-5). This value cannot be explained by the amount of H_2O present in the adsorbed BLA sample, since it is only 6% of the total volume of the NMR sample. Complete amide proton exchange of adsorbed BLA would result in a value of $I(\infty)$ that is 6% of $I(0)$, which is for all observable residues below the noise level of the TOCSY spectra. Consequently, the observed remaining amide proton intensity after 8 minutes of H/D exchange of adsorbed BLA must be due to a population of adsorbed BLA molecules that has a significantly higher amide proton exchange protection than the majority of the adsorbed BLA molecules. This population of approximately 40% of the total amount of adsorbed BLA experiences non-detectable H/D exchange on the time scale of the experiments (i.e. 8 minutes).

Further insight into the conformations of the two populations of adsorbed BLA molecules is obtained by the acquisition of a near-UV circular dichroism (CD) spectrum of BLA adsorbed on polystyrene nanospheres (Fig. 5-6). Near-UV CD spectroscopy mainly informs about the microenvironment of aromatic residues. Despite the large number of scans acquired, this near-UV CD spectrum is rather noisy due to the presence

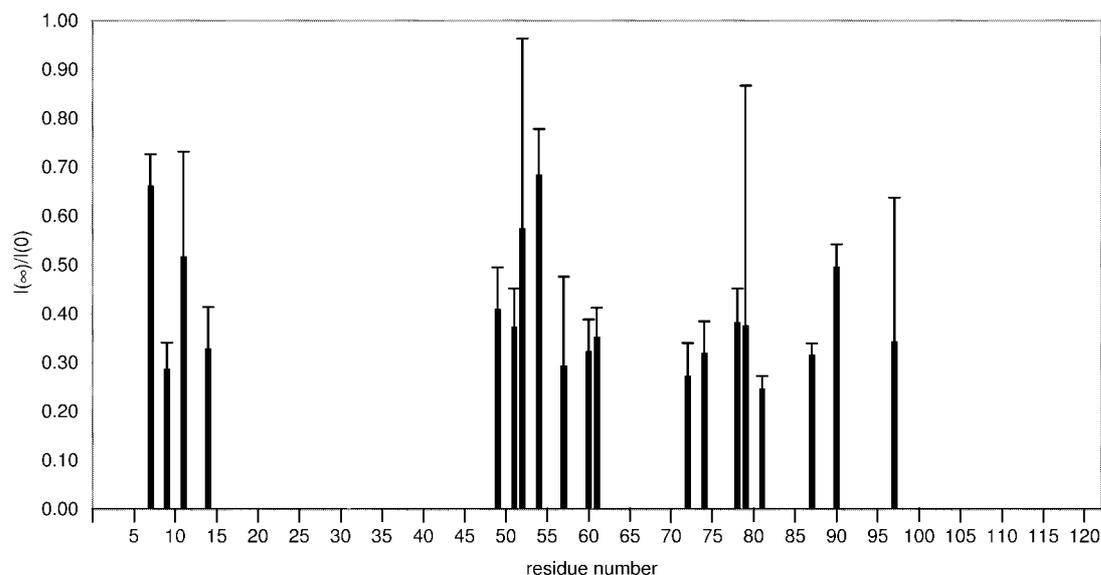


Fig. 5-5. Fraction of the amide proton NMR intensity of the residues of BLA adsorbed on polystyrene nanospheres at pD 5.5 and 20 °C that does not decay during the 8 minute H/D exchange experiments. The fraction is determined by dividing the final intensity $I(\infty)$ of a backbone amide NMR signal by the corresponding initial intensity $I(0)$ obtained from the non-adsorbed BLA reference exchange data. Error bars show the standard errors of the values as calculated from the standard errors of $I(\infty)$ and $I(0)$.

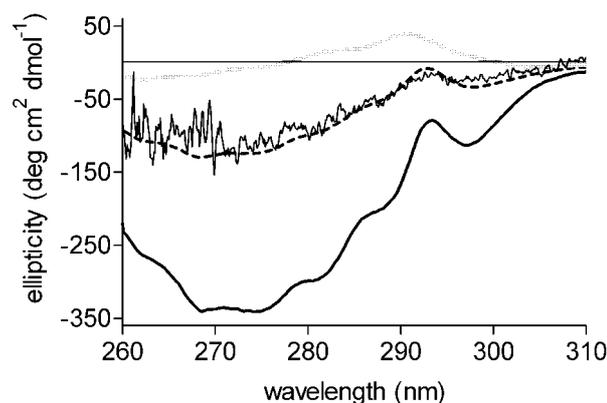


Fig. 5-6. Near-UV CD spectra of native BLA in a 10 mM Tris/HCl buffer at pH 7.5 that contains 1 mM CaCl_2 (thick black line), of BLA in the A-state at pH 2.1 in water (thick grey line), of BLA adsorbed on polystyrene nanospheres in a 10 mM Tris/HCl buffer at pH 7.5 that contains 1 mM CaCl_2 (noisy black line). The simulated near-UV CD spectrum (dashed black line) is composed of 35% of the intensity of the near-UV CD spectrum of native BLA and of 65% of the intensity of the near-UV CD spectrum of BLA in the A-state. The protein concentration ranges between 4.5 and 20 μM . The samples are measured at 20 °C in a 10 mm quartz cuvette.

of the nanospheres. Its features are similar to the spectrum of native BLA, although with significantly reduced absolute ellipticities. The near-UV CD spectrum of adsorbed BLA has increased ellipticities compared to the one of the A-state of BLA, the spectrum of which has ellipticities around zero due to the absence of persistent tertiary structure. Based on the results presented above, it is proposed that the near-UV CD spectrum of

adsorbed BLA represents the combined CD spectra of two populations of adsorbed BLA molecules, each with a different conformation. The main population consists of adsorbed BLA molecules in the A-state (with A-state like H/D exchange rates) and the minor population consists of adsorbed BLA molecules with a native-like near-UV CD spectrum (and with slow H/D exchange rates). Indeed, a simulated spectrum composed of 35% of the intensity of the near-UV CD spectrum of native BLA and of 65% of the intensity of the near-UV CD spectrum of the A-state of BLA practically coincides with the near-UV CD spectrum of BLA adsorbed on polystyrene nanospheres (Fig. 5-6).

5.4 Discussion

In this study, a protein folding intermediate of BLA is induced and simultaneously trapped by spontaneous adsorption of native BLA on a hydrophobic interface. The trapped folding intermediate is characterized by the determination of its backbone amide proton exchange rates, which are expressed as protection factors. The distribution of the protection factors along the primary sequence of adsorbed BLA is similar to the one observed for the A-state of BLA. In addition, far-UV CD and fluorescence spectroscopy show that the adsorbed species has molten globule-like optical spectroscopic characteristics (Engel, et al., 2002). Combined with the detailed study of the amide proton protection factors presented here, it is concluded that the majority (60 - 65%) of the adsorbed BLA molecules are in a partially folded state that has many similarities with the A-state of BLA.

Folding intermediates play an important role in the discussion about the mechanisms involved in protein folding. The acid-denatured molten globule state of BLA (A-state) is a well-documented state and is generally thought to have similar properties as an equilibrium folding intermediate and as a kinetic folding intermediate of BLA (Alexandrescu, et al., 1993; Kuwajima, 1996). The characterization of folding intermediates of most other proteins is difficult because they are marginally stable. The results presented here show that a bovine α -lactalbumin folding intermediate can be successfully trapped by adsorption of BLA on a hydrophobic interface and subsequently studied. Hydrophobic interfaces, like the nanospheres used here, have the potential to be favourably exploited for the spontaneous adsorption of protein states that are characterized by exposed hydrophobic groups, as is shown here to be possible for a molten-globule state. Trapping a folding intermediate by its adsorption on a hydrophobic interface enables the characterization of protein folding intermediates that cannot be studied by conventional methods.

Fourteen residues are identified within adsorbed BLA that have a significantly different protection factor compared to the corresponding ones in the A-state of BLA.

These residues (except R10) are all located in one particular region of adsorbed BLA, which comprises helix C, the calcium-binding site and part of its β -domain (Fig. 5-4). Thus, besides the adsorption-induced conformational change of a native protein to an intermediate state, adsorption also has a local effect on the protection against H/D exchange of the adsorbed protein folding intermediate. It is likely that the adsorbed BLA folding intermediate adapts to the polystyrene interface by local structural changes, e.g. rotation of helix-C, to increase favourable interactions between the adsorbed protein and the polystyrene interface. Such structural changes are not shown in Fig. 5-4.

Is the mechanism of adsorption-induced protein unfolding similar to the one causing global protein unfolding, which is traditionally induced by using a denaturant, pressure or pH? It is shown here, on basis of protection factors, that the adsorbed BLA folding intermediate is oriented with a specific side towards the interface. It seems likely that the adsorption-induced protein unfolding is initiated through local interactions of this side of the protein with the interface. Consequently, the mechanism of adsorption-induced unfolding needs not be similar to the one causing global protein unfolding. Adsorption-induced protein unfolding appears to have similarities to mechanical protein unfolding, during which local interactions also play an important role (Smith, et al., 2003). An example of the latter is protein translocation across cell membranes, where the resistance to mechanical unfolding is determined by local protein structure that experiences a pulling force (Matouschek, 2003). Another indication that adsorption-induced protein unfolding differs from the one causing global protein unfolding comes from the recent finding that the adsorption-induced partial unfolding of BLA to the molten globule state is surprisingly fast, i.e. up to 74 s^{-1} (chapter 2) (Engel, et al., 2002). This is much faster than the rate of unfolding of native BLA to its A-state as induced by traditional methods. A pH jump from pH 5.9 to pH 2.1 results in the formation of the A-state of BLA with an unfolding rate of approximately 10 s^{-1} , as estimated from the apparent half-time of the unfolding kinetics at $25 \text{ }^\circ\text{C}$ (Kuwajima, et al., 1975). In addition, the unfolding of native holo-BLA to its molten globule state by removing the calcium ion through addition of a fluorescent calcium chelator is limited by the calcium off-rate of 5 s^{-1} (Aramini, et al., 1996).

Approximately 60% of the adsorbed BLA molecules has an amide proton exchange behaviour similar to the one of the A-state, while approximately 40% of the adsorbed BLA molecules exhibits much slower H/D exchange than the A-state does. The near-UV CD spectrum of adsorbed BLA supports the presence of these two conformationally different populations of adsorbed BLA molecules. Sixty-five percent of the adsorbed BLA molecules has an A-state-like near-UV CD spectrum and the remaining 35% has a native-like near-UV CD spectrum. These are the most detailed observations of the

phenomenon of structural heterogeneity occurring in a monolayer of adsorbed protein molecules. This structural heterogeneity is explained by the altered conditions experienced by proteins that arrive late at the interface during the adsorption process (Morrissey, 1977; Norde, 1999; Van Tassel, et al., 1997; Zoungrana, et al., 1997). Protein molecules that arrive first at the interface have sufficient space to adapt to the interface, allowing conformational changes like expansion to a molten globule to occur. In contrast, proteins that arrive late at the now crowded interface have no space to optimize their interactions with the interface. Crowding is generally expected to stabilize native protein structure at the expense of less compact non-native structures (Minton, 2000). The observations of slow H/D exchange for 40% of the adsorbed BLA molecules and of native-like CD features of these molecules demonstrate the importance of two-dimensional macromolecular crowding during protein adsorption processes.

5.5 Conclusions

Until now, the detailed conformational properties of protein molecules that are partially unfolded through adsorption on an interface are scarcely studied. In addition, little is known about the mechanisms that dictate unfolding of proteins that are in an adsorbed state. In this study, detailed information about the structure, stability and orientation of a folding intermediate of BLA that is adsorbed on a hydrophobic interface is presented. Adsorption can induce and trap a folding intermediate of BLA on the surface of polystyrene nanospheres. The adsorbed folding intermediate interacts preferentially with the interface via a cluster of residues including those of helix C, the calcium binding site, and part of the β -domain of BLA. Adsorption-induced protein unfolding seems to resemble mechanically induced protein unfolding more than global unfolding of a protein induced by denaturant, pressure or pH. Macromolecular crowding on the solid interface affects the process of adsorption-induced protein unfolding and results in a population of BLA molecules with native-like H/D exchange behaviour. This population (35 to 40% of the total amount of adsorbed BLA) arrived late during adsorption and cannot unfold to a BLA folding intermediate (60 to 65% of the total population) due to macromolecular crowding. The study presented provides new insights in the mechanisms of adsorption-induced protein unfolding. Adsorption of proteins on hydrophobic interfaces can be used as a valuable method to trap and subsequently study otherwise marginally stable protein folding intermediates.

Summary

The adsorption of protein molecules on interfaces is a ubiquitous phenomenon in both natural and man-made systems. Adsorbed protein molecules undergo conformational changes induced by their adsorption on an interface. It is now recognized that these adsorption-induced conformational changes of proteins are an essential part of the process of protein adsorption. However, detailed knowledge of adsorption-induced conformational changes is lacking, which hampers the further understanding of the process of protein adsorption.

The objective of the study presented in this thesis is to obtain detailed information about the conformational changes of bovine α -lactalbumin (BLA) induced by its adsorption on negatively charged, hydrophobic polystyrene nanospheres. This information is obtained by the use of three spectroscopic techniques: fluorescence spectroscopy, circular dichroism (CD) spectroscopy, and Nuclear Magnetic Resonance (NMR) spectroscopy in combination with hydrogen/deuterium (H/D) exchange.

Adsorption induces unfolding of BLA

The addition of BLA molecules in the native state to a suspension of hydrophobic polystyrene nanospheres results in the spontaneous and rapid adsorption of all added protein molecules on the nanosphere surface. CD and fluorescence spectroscopy show that the adsorbed BLA molecules have spectral properties that are characteristic for the molten globule state of BLA, i.e. disordered tertiary structure and native-like secondary structure. It is shown by H/D exchange and NMR spectroscopy that the majority ($\pm 60\%$) of the adsorbed BLA molecules have conformational properties similar to BLA molecules in the acid-denatured state (A-state), a state that is similar to the molten-globule state. A folding intermediate of BLA is induced and trapped by adsorption of the protein on hydrophobic polystyrene nanospheres.

Adsorption-induced unfolding of BLA resembles mechanical unfolding

Several residues, clustered on one side of the adsorbed folding intermediate of BLA, have altered amide proton exchange protection factors compared to those of the A-state of BLA. This side of BLA preferentially interacts with the interface, and includes residues in helix C, the calcium binding site and part of its β -domain. It seems likely that the adsorption-induced protein unfolding is initiated through local interactions of this side of the protein with the interface. In this way, adsorption-induced protein unfolding resembles more the mechanical unfolding of a protein, during which local

interactions also play a role, than the global unfolding of a protein as induced by traditional methods, such as heat, pressure or denaturant-induced unfolding. Another indication that adsorption-induced protein unfolding differs from global protein unfolding comes from the finding that the adsorption-induced partial unfolding of BLA to the molten globule state is surprisingly fast, i.e. up to 74 s^{-1} , which is faster than unfolding induced by pH or a conventional denaturant.

Macromolecular crowding causes structural heterogeneity of adsorbed BLA molecules

Whereas the majority ($\pm 60\%$) of the adsorbed protein molecules has conformational properties similar to BLA molecules in the A-state, the minority ($\pm 40\%$) of adsorbed BLA molecules has significantly lower amide proton exchange rates than the adsorbed BLA folding intermediate has. This minority of BLA molecules arrived at a late stage during the adsorption process and could not adapt to the interface by unfolding to the A-state because two-dimensional macromolecular crowding on the nanosphere surface prevented them to. Two-dimensional macromolecular crowding thus causes structural heterogeneity of the adsorbed BLA molecules on the surface of the polystyrene nanospheres. Crowding is generally expected to stabilize native protein structure at the expense of less compact non-native structures. Near-UV CD spectroscopy confirms the presence of the two structurally different populations of adsorbed BLA molecules in a ratio similar to the one found by NMR spectroscopy and H/D exchange.

Adsorption-induced unfolding of BLA is fully reversible upon displacement

The surfactants CHAPS and Tween 20 are able to displace adsorbed BLA molecules from the surface of the polystyrene nanospheres. CHAPS displaces up to 90% of the adsorbed BLA molecules and is more effective in displacing adsorbed BLA than Tween 20. The displaced BLA molecules refold to native BLA as determined by CD and fluorescence spectroscopy. Two-dimensional $^1\text{H-NMR}$ spectroscopy shows that the chemical shifts of the backbone amide protons of holo-BLA after its adsorption and subsequent displacement from polystyrene nanospheres by the surfactant CHAPS are identical to those of native holo-BLA. Consequently, adsorption-induced unfolding of holo-BLA on polystyrene nanospheres is fully reversible at the residue-level upon displacement of BLA from these nanospheres by the surfactant CHAPS.

At low surfactant concentrations and prolonged incubation, displaced BLA has non-native fluorescence and near-UV CD spectral properties and contains a population of molecules that has higher electrophoretic mobility compared to native BLA. These non-native properties are most likely caused by intramolecular disulfide shuffling initiated

by a few reactive groups on the surface of the nanospheres during prolonged homomolecular exchange of adsorbed BLA.

NMR spectroscopy is a valuable tool to study the adsorption of BLA on suspended nanospheres

The adsorption of apo- and holo-BLA on suspended solid polystyrene nanospheres can be studied by NMR spectroscopy. In spite of the presence of 7% (w/w) solid polystyrene nanospheres that are covered by CHAPS or BLA, the quality of the 1D and 2D ^1H -NMR spectra of non-adsorbed apo- and holo-BLA is surprisingly good, and allows the determination of the three-dimensional structure of non-adsorbed BLA molecules present in the sample.

In contrast to methods based on the physical separation of proteins from suspended solid particles, e.g. ultrafiltration, NMR spectroscopy allows the non-invasive estimation of the amount of proteins adsorbed on these nanospheres, without disturbing the equilibrium of the system, as all components are present during the NMR measurements. The severe broadening of the protein resonances due to the large rotational correlation time of the adsorbed protein molecules is favourably exploited in this method. The results presented show that NMR spectroscopy is clearly feasible to study the adsorption of proteins on suspended solid particles.

Concluding remarks and outlook

The experimental conditions associated with adsorption-induced protein unfolding experiments significantly differ from those associated with traditional unfolding experiments in which protein unfolding is induced by heat, pressure or conventional denaturants. As opposed to traditional protein unfolding experiments, in adsorption-induced protein unfolding experiments the presence of an interface is required, which complicates the experimental study of adsorption-induced protein unfolding. In addition, adsorption-induced protein unfolding is shown here to be only reversible upon addition of a surfactant. Dilution of the protein-adsorbent system does not reverse protein unfolding in contrast to protein unfolding by a conventional denaturant. It is thus impossible to acquire classical equilibrium unfolding curves like those routinely obtained for denaturant-induced protein unfolding. As opposed to protein unfolding by conventional denaturants, which requires high concentrations of the denaturant involved, adsorption-induced protein unfolding, can already be achieved by the addition of only 5 nM of polystyrene nanospheres, as shown here for BLA. Due to these unusual experimental conditions, it is a major challenge to obtain detailed information at the

molecular level about adsorption-induced protein unfolding. It is shown here that the combination of NMR spectroscopy, H/D exchange, CD spectroscopy and (stopped-flow) fluorescence spectroscopy are powerful tools to study the adsorption of proteins on solid interfaces.

The methods for the investigation of protein adsorption presented in this thesis are focused on one protein (BLA) and one surface (polystyrene nanospheres), but should be applicable to the adsorption of other proteins on other surfaces as well. The determination by NMR spectroscopy of the amounts of adsorbed protein on suspended particles is possible with any soluble protein. However, information at the residue level about the conformation of an adsorbed protein requires the protein to be smaller than approximately 40 kD to allow the routine assignment of the backbone amide proton resonances by NMR spectroscopy. In addition, the selected solid adsorbent must form a stable suspension in water, and should provide sufficient surface area so that a substantial amount of protein can be adsorbed. NMR spectroscopy should be powerful as well to study proteins adsorbed on liquid interfaces, like those present in oil/water emulsions, in a manner similar to the one used here for the study of protein adsorption on solid polystyrene nanospheres.

The results presented here show that a bovine α -lactalbumin folding intermediate can be trapped by adsorption of BLA on a hydrophobic interface and that it can be subsequently characterized. Hydrophobic interfaces have the potential to be favourably exploited for the spontaneous adsorption of proteins that are in a state that is characterized by exposed hydrophobic groups, as is the case for the molten-globule state of BLA. Marginally stable protein folding intermediates, which often cannot be studied in solution by conventional methods, can be trapped by their adsorption on hydrophobic interfaces. This could be a valuable method for the study of protein folding intermediates.

Samenvatting (Summary in Dutch)

Gedetailleerde karakterisering van adsorptiegeïnduceerde eiwitontvouwing

De adsorptie van eiwitmoleculen aan grensvlakken is een veelvoorkomend fenomeen in zowel natuurlijke als kunstmatige systemen. Geadsorbeerde eiwitmoleculen ondergaan conformatieveranderingen die worden geïnduceerd door hun adsorptie aan een grensvlak. Het blijkt dat deze adsorptiegeïnduceerde conformatieveranderingen een essentieel deel uitmaken van het adsorptieproces. Gedetailleerde kennis van adsorptiegeïnduceerde conformatieveranderingen ontbreekt echter waardoor het verkrijgen van meer inzicht in het proces van eiwitadsorptie wordt belemmerd.

Het doel van het onderzoek dat in dit proefschrift is beschreven is het verkrijgen van gedetailleerde informatie over conformatieveranderingen van het rundereiwit α -lactalbumine (BLA) die worden geïnduceerd door de adsorptie van dit eiwit op negatief-geladen hydrofobe polystyreenbolletjes. Deze informatie wordt verkregen door gebruik te maken van drie spectroscopische technieken: fluorescentiespectroscopie, circulair dichroïsmespectroscopie (CD), en nucleaire magnetische resonantiespectroscopie (NMR) in combinatie met waterstof/deuterium (H/D) uitwisselingsexperimenten.

Adsorptiegeïnduceerde ontvouwing van BLA

Het toevoegen van BLA-moleculen in de natieve toestand aan een suspensie van hydrofobe polystyreenbolletjes leidt tot de spontane en snelle adsorptie van alle toegevoegde eiwitmoleculen op het oppervlak van de polystyreenbolletjes. CD- en fluorescentiespectroscopie tonen aan dat de geadsorbeerde BLA-moleculen spectrale eigenschappen hebben die karakteristiek zijn voor de *molten globule*-toestand van BLA, een toestand die zich kenmerkt door de aanwezigheid van ongeordende tertiaire structuur en natieve secundaire structuur. H/D uitwisselingsexperimenten en NMR-spectroscopie tonen aan dat de conformatie van de meerderheid ($\pm 60\%$) van de geadsorbeerde BLA-moleculen lijkt op die van BLA-moleculen in de zuurgedenatureerde toestand (*A-state*), een toestand die lijkt op de *molten globule*-toestand. Op deze manier wordt een vouwingsintermediair van BLA geïnduceerd en gefixeerd door adsorptie van het eiwit op hydrofobe polystyreenbolletjes.

Adsorptiegeïnduceerde ontvouwing van BLA lijkt op mechanische ontvouwing

Een aantal residuen, gegroepeerd aan één kant van de geadsorbeerde vouwingsintermediair van BLA, heeft andere *protection factors* (een getal dat de bescherming tegen H/D uitwisseling van de amideprotonen van het BLA-molecuul uitdrukt) dan de *A-state* van BLA. Deze kant van het BLA-molecuul is bij voorkeur in contact met het oppervlak. De residuen van helix C, de calcium-bindingsplaats en een deel van het β -domein liggen in dit deel van het BLA-molecuul. Het is waarschijnlijk dat de adsorptiegeïnduceerde ontvouwing wordt geïnitieerd door lokale interactie van deze kant van het eiwitmolecuul met het oppervlak. Adsorptiegeïnduceerde eiwitontvouwing lijkt zodoende meer op de mechanische ontvouwing van een eiwit, waarbij lokale interacties een rol spelen, dan op globale eiwitontvouwing (ontvouwing waarbij het eiwit als één geheel ontvouwt) zoals die wordt geïnduceerd met conventionele methoden, bijvoorbeeld hitte-, druk- of denaturantgeïnduceerde ontvouwing. Er is nog een indicatie waarom adsorptiegeïnduceerde eiwitontvouwing anders plaatsvindt dan globale eiwitontvouwing. De adsorptiegeïnduceerde ontvouwing van BLA naar een *molten globule*-toestand is namelijk verassend snel, tot 74 s^{-1} , wat sneller is dan ontvouwing geïnduceerd door het veranderen van de pH of door het toevoegen van een conventionele denaturant.

Macromoleculair gedrang veroorzaakt conformationele heterogeniteit van geadsorbeerde BLA-moleculen

Hoewel de meerderheid ($\pm 60\%$) van de geadsorbeerde BLA-moleculen een conformatie heeft die lijkt op die van de *A-state* van BLA, heeft de rest ($\pm 40\%$) van de geadsorbeerde BLA-moleculen hogere *protection factors* dan de geadsorbeerde vouwingsintermediair van BLA. Deze minderheid van BLA-moleculen arriveert laat tijdens het adsorptieproces en kan zich door middel van ontvouwing naar de *A-state* niet aanpassen aan het oppervlak doordat zij worden gehinderd door tweedimensionaal macromoleculair gedrang (een zodanig hoge concentratie van moleculen in een systeem dat de thermodynamische eigenschappen van dat systeem drastisch beïnvloed worden). Tweedimensionaal macromoleculair gedrang veroorzaakt conformationele heterogeniteit in de BLA moleculen die zijn geadsorbeerd op de polystyreenbolletjes. Het wordt doorgaans aangenomen dat macromoleculair gedrang de natieve toestand van een eiwit stabiliseert ten koste van minder-compacte toestanden van een eiwit. Nabij-UV CD-spectroscopie bevestigt de aanwezigheid van twee populaties BLA-moleculen, ieder met een andere conformatie, in een verhouding die overeenkomt met de verhouding zoals die is bepaald met NMR-spectroscopie en H/D uitwisselingsexperimenten.

Adsorptiegeïnduceerde ontvouwing van BLA is volledig reversibel na verdringing van geadsorbeerd BLA van het oppervlak

De detergentia CHAPS en Tween 20 zijn in staat om geadsorbeerde BLA-moleculen van het oppervlak van de polystyreenbolletjes te verdringen. CHAPS verdringt tot 90% van de geadsorbeerde BLA-moleculen, en is effectiever in het verdringen van geadsorbeerd BLA dan Tween 20. CD- en fluorescentiespectroscopie tonen aan dat de verdrongen BLA-moleculen hervouwen naar natief BLA. Tweedimensionale ¹H-NMR-spectroscopie toont aan dat de relatieve resonantiefrequenties van de amideprotonen in de hoofdketen van holo-BLA, nadat het is geadsorbeerd en vervolgens verdrongen van de polystyreenbolletjes door het detergens CHAPS, identiek zijn aan die van natief holo-BLA. Adsorptiegeïnduceerde ontvouwing van holo-BLA is dus volledig reversibel op residu-niveau na verdringing van geadsorbeerd BLA van de polystyreenbolletjes door het detergens CHAPS.

Bij lage detergentiac concentraties en langdurige adsorptie heeft het verdrongen BLA niet-natieve fluorescentie- en CD-spectra en een hogere electroforetische mobiliteit vergeleken met natief BLA. Deze niet-natieve eigenschappen worden hoogstwaarschijnlijk veroorzaakt door intramoleculaire disulfideverschuivingen. Deze verschuivingen kunnen worden geïnitieerd door enkele reactieve groepen op het oppervlak van de polystyreenbolletjes tijdens langdurige homomoleculaire uitwisseling van geadsorbeerde BLA-moleculen.

NMR-spectroscopie is een waardevolle techniek voor de bestudering van de adsorptie van BLA op gesuspendeerde polystyreenbolletjes

De adsorptie van apo- en holo-BLA op gesuspendeerde vaste polystyreenbolletjes kan worden bestudeerd met NMR-spectroscopie. Ondanks de aanwezigheid van 7% (w/w) polystyreenbolletjes die bedekt zijn met CHAPS of BLA is de kwaliteit van de een- en tweedimensionale ¹H-NMR-spectra van vrij BLA verrassend goed. Dit heeft tot gevolg dat de driedimensionale structuur van de niet-geadsorbeerde BLA-moleculen kan worden bepaald in aanwezigheid van deze polystyreenbolletjes.

In tegenstelling tot methoden die gebaseerd zijn op de fysieke scheiding van eiwitten en gesuspendeerde deeltjes, bijvoorbeeld ultrafiltratie, kan met NMR-spectroscopie de hoeveelheid geadsorbeerd eiwit worden bepaald in aanwezigheid van alle betreffende componenten van het systeem, zonder het evenwicht van het systeem te beïnvloeden. Bij deze methode wordt gebruik gemaakt van de extreme lijnverbreding van eiwitresonanties die het gevolg zijn van de lange rotatiecorrelatietijd van de geadsorbeerde eiwitmoleculen. De hier gepresenteerde resultaten tonen aan dat NMR-

spectroscopie een geschikte techniek is voor de bestudering van de adsorptie van eiwitten op gesuspendeerde vaste deeltjes.

Conclusies en vooruitzichten

De experimentele omstandigheden tijdens adsorptiegeïnduceerde eiwitontvouwings-experimenten verschillen nogal van die gedurende traditionele ontvouwingsexperimenten, waarbij eiwitontvouwing wordt geïnduceerd door hitte, druk of een conventionele denaturant. In tegenstelling tot deze traditionele ontvouwingsexperimenten is bij adsorptiegeïnduceerde eiwitontvouwing de aanwezigheid van een oppervlak vereist, wat de experimenten bemoeilijkt. Bovendien is adsorptiegeïnduceerde eiwitontvouwing alleen reversibel als een detergent wordt gebruikt om het geadsorbeerde eiwit te verdringen, zoals in dit proefschrift is aangetoond. Het verdunnen van het gebruikte eiwit-polystyreenbolletjes-systeem leidt niet tot hervouwing van het geadsorbeerde eiwit, in tegenstelling tot eiwitontvouwing geïnduceerd met een conventionele denaturant. Hierdoor is het onmogelijk om klassieke evenwichtsontvouwingscurves te verkrijgen zoals dat routinematig gebeurt voor denaturantgeïnduceerde eiwitontvouwing. Adsorptiegeïnduceerde eiwitontvouwing kan al optreden als slechts 5 nM polystyreenbolletjes aan een eiwitoplossing worden toegevoegd, wat sterk contrasteert met de hoge denaturantconcentraties die nodig zijn bij conventionele denaturantgeïnduceerde eiwitontvouwing. Vanwege deze ongebruikelijke experimentele omstandigheden is het een aanzienlijke uitdaging om gedetailleerde informatie op moleculair niveau over adsorptiegeïnduceerde eiwitontvouwing te verkrijgen. In dit proefschrift is aangetoond dat CD-spectroscopie, fluorescentiespectroscopie en de combinatie van NMR-spectroscopie met H/D uitwisselingsexperimenten krachtige technieken zijn om de adsorptie van eiwitten op vaste oppervlakken te bestuderen.

De hier beschreven methoden voor het onderzoek naar eiwitadsorptie concentreren zich op één eiwit (BLA) en één oppervlak (polystyreenbolletjes), maar zijn in principe ook toepasbaar op de adsorptie van andere eiwitten op andere oppervlakken. De bepaling van de hoeveelheid geadsorbeerd eiwit op gesuspendeerde deeltjes met behulp van NMR-spectroscopie is mogelijk voor elk willekeurig, oplosbaar eiwit. Voor het bepalen van de conformatie op residu-niveau van een geadsorbeerd eiwit met behulp van de routinematige toekenning van de resonantiefrequenties van amideprotonen in de hoofdketen van het eiwit, is het noodzakelijk dat het eiwit kleiner is dan ongeveer 40 kD. Daarnaast moeten de gekozen deeltjes een stabiele suspensie vormen en voldoende oppervlak bieden voor de adsorptie van een aanzienlijke hoeveelheid eiwit. NMR-spectroscopie zou ook gebruikt kunnen worden voor de bestudering van eiwitten

geadsorbeerd op vloeibare grensvlakken, zoals die voorkomen in olie/water emulsies, op een zelfde manier zoals die hier is gebruikt voor de bestudering van eiwitadsorptie op vaste polystyreenbolletjes.

De resultaten die in dit proefschrift zijn gepresenteerd tonen aan dat een vouwingsintermediair van BLA kan worden gefixeerd door adsorptie van BLA op een hydrofoob oppervlak. Dit maakt het vervolgens mogelijk om deze geadsorbeerde vouwingsintermediair te karakteriseren. Hydrofobe oppervlakken kunnen benut worden voor de spontane adsorptie van eiwitten die worden gekenmerkt door onbeschermde hydrofobe groepen, zoals de *molten globule*-toestand van BLA. Vouwingsintermediaren met een lage stabiliteit, waarvan de bestudering in oplossing met conventionele methoden vaak niet mogelijk is, kunnen worden gefixeerd door adsorptie op hydrofobe oppervlakken. Deze methode biedt perspectief voor de gedetailleerde bestudering van dergelijke vouwingsintermediaren.

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List of abbreviations

| | |
|----------|--|
| BLA | bovine α -lactalbumin |
| CD | circular dichroism |
| CHAPS | 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| cmc | critical micelle concentration |
| DQF-COSY | double quantum filtered correlation spectroscopy |
| DSS | 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt |
| EDTA | ethylenediaminetetraacetic acid |
| FID | free induction decay |
| GdnHCl | guanidine hydrochloride |
| ICP-OES | inductively coupled plasma optical emission spectrometry |
| MG | molten globule |
| NMR | nuclear magnetic resonance |
| NOESY | nuclear Overhauser effect spectroscopy |
| NS | nanospheres |
| PAGE | polyacrylamide gel electrophoresis |
| pD | glass electrode reading of the pH meter, uncorrected for isotope effects |
| TOCSY | total correlation spectroscopy |
| TRIS | 2-amino-2-(hydroxymethyl)-1,3-propanediol |

About the author

Maarten Frans Maria Engel was born in Zevenaar, The Netherlands, on 3 May 1971. He completed High School (“Atheneum”) at Isala College in Silvolde in 1989. In September of the same year, he started his graduate studies at the Faculty of Chemical Engineering at Twente University in Enschede. His main subjects were Polymer Chemistry, Physical Chemistry, Biomedical Materials Science, and Colloid and Interface Chemistry. In February 1995, he obtained his MSc-degree after successfully completing a 10-month project entitled “Antifouling coatings based on copper release from a polyanhydride matrix” in the Department of Polymer Chemistry and Biomaterials (Prof. J. Feijen). In March 1995, he joined the Agrotechnological Research Institute (ATO-DLO) in Wageningen as part of a compulsory 1-year period of civil service. There he studied protein processing for non-food applications, and continued this research until the beginning of 1999. In April 1999, the author started his PhD research on a project entitled “Conformational changes of proteins at interfaces” in the Department of Biochemistry at Wageningen University, under the supervision of Dr. C.P.M. van Mierlo and Prof. A.J.W.G. Visser. Since February 2004, he has been doing post-doctoral research into the interaction of Islet Amyloid Polypeptide with membranes. This research is performed in the Department of Metabolic and Endocrine Diseases at the University Medical Centre in Utrecht (Dr. J.W.M. Höppener), and in the Department of Biochemistry of Membranes at Utrecht University (Prof. J.A. Killian).

Publications

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