Protein Exposed Hydrophobicity Reduces the Kinetic Barrier for Adsorption of Ovalbumin to the Air−Water Interface

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Received May 20, 2003. In Final Form: July 17, 2003

Using native and caprylated ovalbumin, the role of exposed hydrophobicity on the kinetics of protein adsorption to the air−water interface is studied. First, changes in the chemical properties of the protein upon caprylation were characterized followed by measurement of the changes in adsorption kinetics. No change in the molecular structure of ovalbumin was observed upon caprylation. However, aggregation of the protein was observed when more than three capryl chains were coupled per protein. A batch of caprylated ovalbumin with an average coupling of four capryl chains per protein was separated into a monomeric and an aggregated protein fraction. The exposed hydrophobicity of the monomeric and the aggregated species was measured using 8-anilino-1-naphthalenesulfonic acid fluorescence. The exposed hydrophobicity of the monomeric fraction was significantly higher than that of the nonmodified protein. The changes in adsorption kinetics were studied by measuring the increase in surface load (Γ) and in surface pressure (Π) as a function of time (*t*) using an ellipsometer and a Wilhelmy plate, respectively. It was found that the increase of surface load in time (even at low surface coverage) is much lower than the value that was calculated from diffusional transport. This shows that the adsorption of native ovalbumin is barrier limited. The adsorption kinetics of the caprylated protein follow the calculations from diffusional transport more closely, which shows that the energy barrier for adsorption of caprylated ovalbumin is much lower than for the native protein. The surface pressure at a certain surface load (Π−Γ) was not affected by the modification, indicating that the effect of increased hydrophobicity is limited to the adsorption process.

1. Introduction

Protein adsorption to the air−water interface leads to the formation of an interfacial network that changes the physical and mechanical properties of that interface. This interfacial network is necessary to form and stabilize foams from protein solutions upon aeration. While the concentration of protein in the adsorbed surface layer and the resulting surface pressure—in equilibrium—are comparable for many protein solutions, large differences are found in their ability to form and/or stabilize foam. For instance, ovalbumin is a protein that has surface rheological properties that are comparable to those of β-lactoglobulin.[1] However, it was found that no foam could be formed with ovalbumin at protein concentrations between 0.01 and 3.0 mg/mL, while for β-lactoglobulin such concentrations were sufficient to obtain foam.[1] This poor foaming behavior of ovalbumin was suggested to be related to slow adsorption kinetics, which in turn are determined by the molecular properties of the protein.

A thorough description of protein adsorption to air−water interfaces is given by MacRitchie and Alexander[2,3] and Graham and Philips.[4] Adsorption starts with diffusion of proteins from the bulk to the interface. Close to the interface the protein can go from the "dissolved" to the adsorbed state. The main drive for this adsorption is the decrease of exposure of hydrophobic groups to the aqueous medium.[5] On the basis of the assumption that protein adsorption is essentially irreversible, proteins are assumed to adsorb without an energy barrier. This means that all the proteins that come into contact with the surface adsorb and that the adsorption is only limited by diffusional (or convectional) transport of proteins to the surface.[4−6] Only at higher surface load the protein needs to overcome an energy barrier, related to the work required to adsorb against the existing surface pressure and the chance for the adsorbing protein to arrive at an empty location at the interface.[2,7] However, in some cases indications were found that the adsorption of proteins was slower than predicted from the diffusional transport, although this was not directly attributed to the existence of an energy barrier to adsorption. For instance, Damodaran et al.

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(1) Martin, A. H.; et al. Network forming properties of various proteins adsorbed at the air−water interface in relation to foam stability. *J. Colloid Interface Sci.* **2002**, *254*, 175−183.

(2) MacRitchie, F.; Alexander, A. E. Kinetics of adsorption of proteins at interfaces, Part I. The role of bulk diffusion in adsorption. *J. Colloid Interface Sci.* **1963**, *18*, 453−457.

(3) MacRitchie, F.; Alexander, A. E. Kinetics of adsorption of proteins at interfaces, Part II. the role of pressure barriers in adsorption. *J. Colloid Interface Sci.* **1963**, *18*, 458−436.

(4) Graham, D. E.; Phillips, M. C. Proteins at Liquid Interfaces, I: Kinetics of Adsorption and Surface Denaturation. *J. Colloid Interface Sci.* **1979**, *70*, 403−414.

(5) Narsimhan, G.; Uraizee, F. Kinetics of Adsorption of Globular-Proteins at an Air-Water- Interface. *Biotechnol. Prog.* **1992**, *8*, 187−196.

(6) Guzman, R. Z.; Carbonell, R. G.; Kilpatrick, P. K. The adsorption of proteins to gas−liquid interfaces. *J. Colloid Interface Sci.* **1986**, *114*, 536−547.

(7) Eastoe, J.; Dalton, J. S. Dynamic surface tension and adsorption mechanisms of surfactants at the air−water interface. *Adv. Colloid Interface Sci.* **2000**, *85*, 103−144.

10.1021/la034868p CCC: $25.00 © 2003 American Chemical Society
Published on Web 09/03/2003

studied the adsorption of BSA variants in different states of unfolding and found that all unfolded variants adsorbed faster than the native protein.[8] Their conclusion was that the treatment resulted in differences in the rates of adsorption and desorption which had to be related to solvent−solute interactions. A similar conclusion was made by Beverung et al.,[9] who studied the adsorption of ovalbumin at the heptane−water and air−water interfaces. They observed a large difference in the induction times for the surface pressure of the heptane−water and air−water interfaces (10 and 1000 s, respectively). This difference was proposed to be due to the greater affinity of hydrophobic groups of ovalbumin for the heptane than the air. From this work already an indication was obtained for the role of exposed hydrophobicity in adsorption.

Other studies on the role of protein hydrophobicity were based on the comparison of the adsorption of different proteins. Due to the fact that the proteins studied differed in other structural properties as well (e.g., electrostatic charge, size), it appeared to be difficult to derive conclusions on the influence of hydrophobicity on adsorption kinetics.[10,11]

A more controllable way is to chemically modify proteins to increase the exposed hydrophobicity. If the reaction conditions are chosen well, not only the primary structure but also the other structural properties of the native and the modified protein will be the same. In this way, experimental results can be specifically attributed to the chemically modified property. A commonly used technique to increase protein exposed hydrophobicity is the reaction of *N*-hydroxysuccinimidic esters of fatty acids with accessible amino groups of a protein.[12−15] Using this technique, fatty acids of different chain lengths have been successfully coupled to different proteins. The extent of acylation is controlled by varying the ratio of protein to activated fatty acid. A review on the interfacial properties of such modified biomolecules is given by Magdassi et al.[16] When chemical lipophilization is used to study processes at the interface, care must be taken to avoid unwanted changes in protein chemical and structural properties, such as unfolding or aggregation of the protein.[12,15,17,18]

Using chemical modification, Baszkin et al.[19] showed that an increase in exposed hydrophobicity of human immunoglobulin G resulted in a faster adsorption. This was based on the measurement of the increase of surface pressure in time, since the actual increase of surface load was only measured for the native protein. This directly shows the importance of a good experimental setup in the study of interfacial properties of proteins. In this work simultaneous measurements of surface load and surface pressure, during the adsorption of native and chemically modified ovalbumin to a clean air−water interface, were used to determine the direct influence of protein exposed hydrophobicity on the adsorption kinetics.

2. Materials and Methods

Isolation of Ovalbumin from Hen Eggs. Ovalbumin was isolated as described by Vachier et al.[20] and Takahashi et al.,[21] from fresh hen eggs (less than 3 h old) that were obtained from the Department of Animal Sciences (Wageningen University and Research Centre). Egg white was manually separated from the yolk and pooled. The egg white was diluted two times with 50 mM Tris (pH 7.5) containing 10 mM β-mercaptoethanol. The solution was gently stirred for 6 h at 4 °C. After centrifugation the supernatant was diluted two times with 50 mM Tris (pH 7.5). Subsequently DEAE Sepharose CL-6B was added to the albumin solution (9 g of DEAE per 100 mL) and the solution stirred gently overnight at 4 °C. The material was transferred onto a glass filter (G2) and subsequently washed with distilled water (1.8 L per 500 g of DEAE) and 0.1 M NaCl (0.5 L per 500 g of DEAE). The bound ovalbumin was eluted from the ion-exchange material with 0.15 M NaCl. The eluate was dialyzed, lyophilized, and stored at −20 °C. The purity of the ovalbumin was found to be 98% (±0.5%) as determined by densitometer analysis of SDS−PAGE gels.

Lipophilization of Ovalbumin with Succinimidic Esters of Capric Acid. *Esterification of Capric Acid with N-Hydroxysuccinimide*. To increase the reactivity of capric acid toward protein amino groups, the fatty acid was first esterified with *N*-hydroxysuccinimic acid as described by Lapidot et al.[22] Esterification was realized by reaction of equimolar amounts of fatty acid and *N*-hydroxysuccinimide in anhydrous tetrahydroxyfuran, in the presence of 1,3-dicyclohexylcarbodiimide for 36 h at room temperature. The precipitated side product dicyclohexylurea was removed by filtration, and the filtrate was dried in a vacuum evaporator. To remove other impurities, the succinimidic ester of capric acid ($C_{10:0}$-SU) was redissolved (1 g in 5 mL) in 60% ethanol in H_2O at 60 °C and recrystallized by cooling to room temperature. The yield of the esterification reaction was approximately 70%. The purity and identity of the activated capric acid were confirmed (>97% purity) using thin-layer chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy (results not shown).

Modification of Ovalbumin. Activated capric acid ($C_{10:0}$-SU) was covalently coupled to the free primary amino groups of ovalbumin as described by Liu et al.[23] To obtain ovalbumin with different degrees of modification, four batches were prepared that differed in the added amount of $C_{10:0}$-SU. Ovalbumin was dissolved at a concentration of 8.5 mg/mL in 250 mL of 100 mM sodium carbonate buffer (pH 8.5). $C_{10:0}$-SU acid was dissolved at concentrations of 0.4, 1.4, 2.8, and 8.0 mg/mL in 40 mL of DMSO

(8) Damodaran, S.; Song, K. B. Kinetics of adsorption of proteins at interfaces: role of protein conformation in diffusional adsorption. *Biochim. Biophys. Acta* **1988**, *954*, 253−264.

(9) Beverung, C. J.; Radke, C. J.; Blanch, H. W. Protein adsorption at the oil/ water interface: characterization of adsorption kinetics by dynamic interfacial tension measurements. *Biophys. Chem.* **1999**, *81*, 59−80.

(10) Tripp, B. C.; Magda, J. J.; Andrade, J. D. Adsorption of Globular-Proteins at the Air-water Interface as Measured Via Dynamic Surface-Tension - Concentration- Dependence, Mass-Transfer Considerations, and Adsorption- Kinetics. *J. Colloid Interface Sci.* **1995**, *173*, 16−27.

(11) Nakai, S.; Li Chan, E.; Hayakawa, S. Contribution of protein hydrophobicity to its functionality. *Nahrung* **1986**, *30*, 327−336.

(12) Haque, Z.; Kito, M. Lipophilization of alphas1-casein. III. Purification and physicochemical properties of novel amphipathic fatty acyl peptides. *J. Agric. Food Chem.* **1984**, *32*, 1392−1397.

(13) Haque, Z.; et al. Lipophilization of soybean glycinin: covalent attachment to long chain fatty acids: effect on hydrophobicity, conformation and surface functional properties. *Agric. Biol. Chem.* **1982**, *46*, 597−599.

(14) Kito, M., Chemical and physical lipophilization of proteins. *J. Am. Oil Chem. Soc.* **1987**, *64*, 1676−1681.

(15) Akita, E. M.; Nakai, S. Lipophilization of beta-lactoglobulin: effect on hydrophobicity, conformation and surface functional properties. *J. Food Sci.* **1990**, *55*, 711−717.

(16) Magdassi, S.; Kamyshny, A.; Baszkin, A. Interfacial properties of hydrophobically modified biomolecules: Fundamental aspects and applications. *J. Dispersion Sci. Technol.* **2001**, *22*, 313−322.

(17) Haque, Z.; Kito, M. Lipophilization of alphas1-casein. I. Covalent attachment of palmitoyl residue. *J. Agric. Food Chem.* **1983**, *31*, 1225−1230.

(18) Kamyshny, A.; Magdassi, S. Hydrophobically modified human IgG: surface and biological activities. *Colloids Surf., B* **1997**, *9*, 147−155.

(19) Baszkin, A.; et al. Native and hydrophobically Modified Human Immunoglobulin G at the Air-water Interface, Sequential and Competitive Adsorption. *J. Colloid Interface Sci.* **2001**, *239*, 1−9.

(20) Takahashi, N.; et al. Role of the intrachain disulfide bond of ovalbumin during conversion into S-ovalbumin. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1464−1468.

(21) Vachier, M. C.; Piot, M.; Awade, A. C. Isolation of Hen Egg-White Lysozyme, Ovotransferrin and Ovalbumin, Using a Quaternary Ammonium Bound to a Highly Cross- Linked Agarose Matrix. *J. Chromatogr., B* **1995**, *664*, 201−210.

(22) Lapidot, Y.; Rappoport, S.; Wolman, Y. Use of esters of N-hydroxysuccinimide in the synthesis of N-acylamino acids. *J. Lipid Res.* **1967**, *8*, 142−145.

(23) Liu, S. T.; et al. Lipophilization of lysozyme by short and middle chain fatty acids. *J. Agric. Food Chem.* **2000**, *48*, 265−269.

and subsequently added to the ovalbumin solution. This gave end concentrations of 0.2 mM ovalbumin and 0.2, 0.7, 1.4, and 4 mM activated capric acid. The mixtures were incubated for 18 h at 40 °C. After incubation the mixtures were dialyzed against water and after lyophilization stored at −20 °C.

Detection of Primary Amino Groups. Primary amino groups in native and caprylated ovalbumin were detected using *o*-phthalic dialdehyde (OPA) as described by Church et al.[24] The OPA reagent was freshly prepared by dissolving 40 mg of OPA in 1 mL of methanol, followed by the addition of 25 mL of 0.1 M sodium borate, 200 mg of 2-(dimethylamino)ethanethiol hydrochloride (DMA), and 5 mL of 10% SDS. The total volume was adjusted to 50 mL with H_2O. Samples were prepared (in triplicate) by adding 65 μL of a 0.1 mM protein solution to 3 mL of the reagent solution. After addition of the reagent solution the samples were left to equilibrate for 2 min. The presence of alkylisoindole derivatives formed after reaction of OPA with free amino groups was measured by the absorbance at 340 nm. To calculate absolute amounts of primary amino groups per protein molecule, a calibration curve was measured using leucine as a reference compound.

Quantification of Protein Surface Hydrophobicity. ANSA (8-anilino-1-naphthalenesulfonic acid) was used in a fluorimetric assay to quantify the protein exposed hydrophobicity of native and caprylated ovalbumin. Ovalbumin (2.3 μM) and ANSA (2.4 mM) were separately dissolved in 10 mM sodium phosphate buffer at pH 7.0. The ANSA solution was titrated in aliquots of 10 μL to 1 mL of ovalbumin solution, as described by Haskard[25] or Alizadeh.[26] Excitation was at 385 nm, and the emission spectrum was measured from 440 to 650 nm on a Perkin-Elmer luminescence spectrometer (LS 50 B) with a scan speed of 120 nm/min. The excitation and emission slits were set at 5 nm, and the measurements were done at 20 °C. The relative apparent hydrophobicity of each sample was expressed as the maximum area of the fluorescence spectrum in the range mentioned above, relative to the maximum area of ANSA fluorescence measured for native ovalbumin.

Determination of Secondary Structure. Samples were dissolved (0.1 mg/mL) in a 10 mM phosphate buffer at pH 7.0. Far-UV CD spectra (190−260 nm) were recorded 8-fold using a 1 mm quartz cuvette on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) and averaged. Spectra were measured at both 20 and 90 °C, with a scan speed of 100 nm/min, a data interval of 0.2 nm, a bandwidth of 1.0 nm, and a response time of 0.125 s. All spectra were corrected for the corresponding protein-free sample and analyzed for the secondary structure estimates using a nonlinear least-squares fitting procedure with reference spectra as described by de Jongh et al.[27]

Evaluation of Tertiary Structure. Intrinsic fluorescence of the tryptophan and tyrosine residues of 0.1 mg/mL protein solutions in 10 mM phosphate buffer (pH 7.0) was measured on a Perkin-Elmer luminescence spectrometer (LS 50 B). The excitation and emission slits were set at 5 nm. The excitation wavelength was 295 or 274 nm to excite tryptophan or tyrosine, respectively. The emission spectra were recorded from 300 to 450 nm with a scan speed of 120 nm/min. Each spectrum was the average of two scans and corrected for a protein-free sample.

Determination of Quaternary Structure. Protein samples were dissolved (5 mg/ mL) in 10 mM phosphate buffer (pH 7.0) in the presence of 100 mM NaCl to avoid nonspecific binding of the protein to the column material. This buffer was also used to equilibrate the Superdex 200 HR column (60 × 1 cm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Samples of 0.2 mL were applied to the column and eluted at a flow rate of 0.4 mL/min with the same buffer, while the eluate was monitored at 280 nm. The column was calibrated for apparent molecular mass determination by applying a mixture of ferritin (440 kDa), aldolase (158 kDa), and ovalbumin (43 kDa).

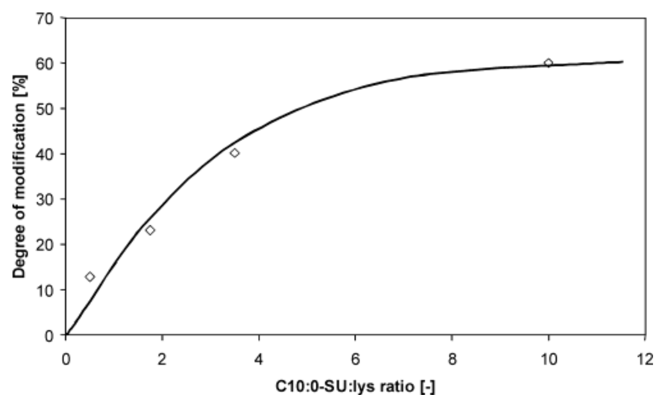

Figure 1. Degree of modification (reproducibility ±4%) as function of the molar ratio of the added reactant ($C_{10:0}$-SU) to the total amount of accessible amino groups (NH_2) in ovalbumin, as determined by the OPA method. The degree of modification is expressed as the percent of the total number of amino groups per protein that has reacted to capric acid.

Adsorption Kinetics Measurements. Adsorption of native and caprylated ovalbumin from bulk solution (0.1 mg/mL, phosphate buffer (10 mM, pH 7.0)) to the air−water interface was studied using a combination of a multiskop ellipsometer (Optrell, Germany) and a Langmuir trough (Riegler and Kirstein, Germany). The trough volume was 100 mL, and the surface area could be varied from 192 to 30 cm^2. To start the experiment with an interface essentially free of adsorbed molecules, the interfacial layer was removed using a custom-made suction device, after which the clean interface was expanded to the maximum area in 40 s (from 30 to 190 cm^2). The adsorption of proteins from the bulk solution (at 20 °C) was measured using the ellipsometer and a Wilhelmy plate. A good description of the ellipsometry technique with further references is given by Graham and Philips.[28] The values for Δ and ψ from the ellipsometer were used to calculate the adsorbed amount of proteins, using a three-layer model, with $n_{air} = 1.000$, $n_{protein\ soln} = 1.3327$, and $dn/dc = 0.18$[29] (the refractive indices of air and water and the refractive index increment of the protein, respectively). The angle of incidence was 50°.

3. Results

Degree of Modification. Lipophilization of ovalbumin was performed at four different protein to substrate (succinylated capric acid) ratios. The average number of capric acid chains that were covalently bound to the amino groups of ovalbumin was determined using a chromophoric assay (the OPA method), based on the selective reaction of *o*-phthalic dialdehyde to amino groups. In Figure 1 it is shown that the degree of modification increases with an increase in the reactant to substrate ratio. The degree of modification levels off at approximately 60%, which corresponds to an average coupling of 12 capric acid chains per ovalbumin molecule. Batra et al.[30] reported that for ovalbumin 15 of the 20 lysine groups are easily modified via succinylation or acylation as a result of their solvent accessibility. That here only 12 groups were modified at the highest excess reagent concentration might have been caused by aggregation of the proteins (as will be discussed

(24) Church, F. C.; et al. Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *J. Dairy Sci.* **1983**, *66*, 1219−1227.

(25) Haskard, C. A.; Li-Chan, E. C. Y. Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS(-)) fluorescent probes. *J. Agric. Food Chem.* **1998**, *46*, 2671−2677.

(26) Alizadeh-Pasdar, N.; Li-Chan, E. C. Y. Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *J. Agric. Food Chem.* **2000**, *48*, 328−334.

(27) Dejongh, H. H. J.; Goormaghtigh, E.; Killian, J. A. Analysis of Circular-Dichroism Spectra of Oriented Protein- Lipid Complexes - toward a General Application. *Biochemistry* **1994**, *33*, 14521−14528.

(28) Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces, II: Adsorption isotherms. *J. Colloid Interface Sci.* **1979**, *70*, 415−426.

(29) Longsworth, E. G. The specific refractive index of some purified proteins. *J. Am. Oil Chem. Soc.* **1948**, *70*, 2719−2724.

(30) Batra, P. B.; Roebuck, M. A.; Uetrecht, D. Effect of Lysine Modification on the Secondary Structure of Ovalbumin. *J. Protein Chem.* **1990**, *9*, 37−44.

Table 1. Controllability of the Lipophilization of Ovalbumin and the Changes in p*I* and Relative Hydrophobicity as Functions of the Degree of Modification

$C_{10:0}$-SU:Lys ratio (mol:mol)	mol of capric acid/ mol of ovalbumin (±1)	degree of modification (%) (±5)	P*I* (measured)	relative hydrophobicity[a] (±0.3)
0	0	0	4.51	1.0
0.5	2	12	4.48	2.4
1.75	5	23	4.45	3.5
3.5	8	40	4.38	6.0
10	12	60	4.3	8.0

[a] The relative hydrophobicity is expressed as the maximal area under the fluorescence spectrum, where the value for native ovalbumin is set at 1.

later), resulting in a decreased accessibility of some of the lysine groups.

Other techniques that have been used to measure the degree of modification are isoelectric focusing (IEF) and mass spectrometry. Results of the IEF are shown in Table 1. A small decrease in p*I* with increasing degree of modification is found (from 4.5 to 4.3). This is expected since the coupling with capric acid eliminates the positive charges of the amino groups of lysine. Calculation of the degree of modification based on the shift of p*I* is not accurate since the p*I* of the protein is close to the pK_a value of the carboxylate groups and therefore rather insensitive to changes. However, since the apparent p*I* is lowered only slightly upon modification, no major influence as a result of changes in the surface charge can be expected. A more direct technique to measure the covalent binding of capric acid to the protein is mass spectrometry. However, due to the low polarizability of the caprylated ovalbumin, no conclusive results could be obtained using this technique.

Exposed Hydrophobicity. The relative exposed hydrophobicity of the proteins was measured by ANSA fluorescence. The total fluorescence intensity of the probe depends on the polarity of its environment and increases with increasing hydrophobicity.[31] ANSA was titrated to protein solutions in 10 μL aliquots, and a fluorescence spectrum was recorded after each addition. The area under the fluorescence spectra was plotted against the added concentration of ANSA (see Figure 2A). At low ANSA concentrations the fluorescence increases with the added amount, but the fluorescence signal levels off at higher ANSA concentrations. This maximum value of each titration curve was taken as a measure of the apparent hydrophobicity of the sample, since no indications of quenching were present in the data. The relative hydrophobicity (shown in Figure 2B) was then calculated by dividing the maximal fluorescence of each sample by that found for native ovalbumin. Similar results were obtained when the initial slope of the fluorescence against the concentration of ANSA was taken as a measure of hydrophobicity (data not shown). The exposed hydrophobicity of caprylated ovalbumin was found to increase linearly with the number of capryl chains on the protein surface, with an exposed hydrophobicity 8 times that of native ovalbumin for the sample with 60% modification.

To distinguish between the effects of the lipophilization process and the presence of coupled capryl chains on the protein, a sample of ovalbumin that was treated in the same way as the lipophilized protein without the addition of activated capric acid was included in the measurements. The exposed hydrophobicity of this nonmodified protein was similar to that of the native protein, indicating that the changes in the hydrophobicity upon lipophilization are due to the presence of the coupled capryl chains.

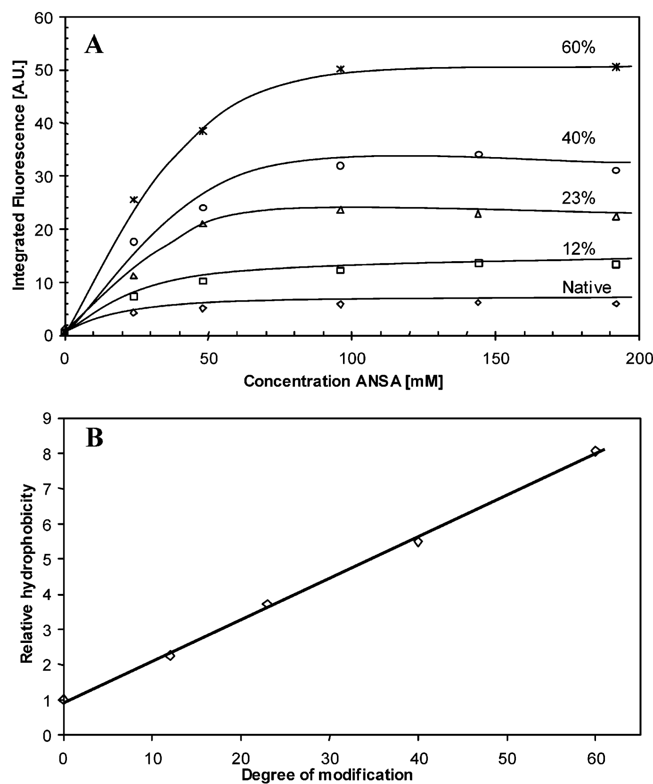

Figure 2. (A) Exposed hydrophobicity of native (◇) and caprylated (□, 12%; ○, 23%; △, 40%; *, 60%) ovalbumin as measured by ANSA fluorescence, fluorescence intensity against added amount of ANSA (protein concentration 2.3 μM, $T = 20$ °C, pH 7.0). (B) Relative hydrophobicity as a function of the degree of modification.

Secondary and Tertiary Structure. To test the effect of the modification procedure on other properties of the protein, the structural properties of modified and nonmodified material were determined. At 20 °C no significant differences were found between the far-UV CD spectra of native and caprylated ovalbumin (results not shown). Secondary structure estimates (all ±2%) derived from spectral analysis indicated a content of 15% α-helix, 54% β-structure (β-helix and β-turn), and 31% random coil for native and caprylated ovalbumin (12%, 23%, and 40%). The intrinsic fluorescence of tryptophan and tyrosine residues in the protein also showed no significant change upon lipophilization, indicating that the tertiary fold of the protein was preserved (results not shown). Only the 60% caprylated sample showed minor differences in far-UV CD and fluorescence spectra, suggestive for a loss of globular packing for a minor part of the protein.

Quarternary Structure. In Figure 3, the size-exclusion chromatograms of the ovalbumin samples with

(31) Tsutsui, T.; Chan, E. Li; Nakai, S. A simple fluorometric method for fat-binding capacity as an index of hydrophobicity of proteins. *J. Food Sci.* **1986**, *51*, 1268−1272.

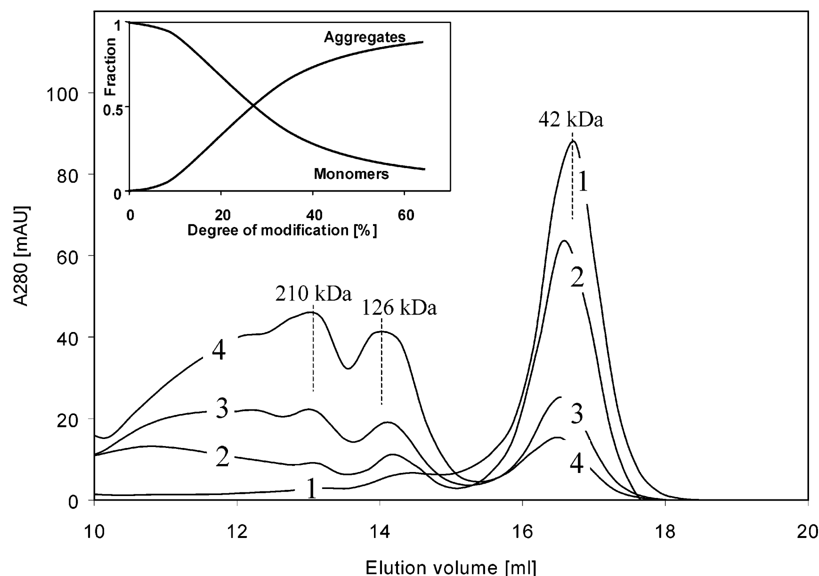

Figure 3. Size exclusion (Superdex-200) chromatograms of caprylated ovalbumin (labels 1–4, 12%, 23%, 40%, and 60%, respectively) (500 μL, 0.1 mg/mL, pH 7.0). Inset: relative amount of aggregates and monomers as a function of the degree of modification.

different degrees of modification are shown. The total amount of protein material, as calculated by integrating the area under the chromatogram, is constant for all samples, indicating that all material elutes from the column. With increasing degree of modification, the fraction of monomeric protein decreases, while the fraction of aggregated protein increases (as shown in the inset). When the average degree of modification exceeds 10% (i.e., three capryl chains per protein), significant aggregation occurs. In the aggregated fraction, at least two distinct peaks can be identified. These correspond with trimeric and pentameric forms of ovalbumin. The maximum size of the higher aggregates does not exceed 600 kDa, which indicates that aggregation stops when approximately 15 proteins are present in the aggregate. To test whether the aggregation is an equilibrium process, both the monomeric and the aggregated fractions were pooled and reapplied to the column. No aggregation of the monomeric fraction was observed, and no disintegration of the aggregates in the aggregated fraction was found (data not shown). This indicates that the aggregation is a nonequilibrium reaction under the conditions used here.

Selection of Sample. To study the effect of increased exposed hydrophobicity on the adsorption kinetics of ovalbumin, a batch with an average coupling of five capryl chains per protein was separated into a monomeric fraction and an aggregated fraction. The (average) degrees of modification were 4 and 7 and the relative hydrophobicities were 1.4 and 5.8 for the monomeric and aggregated fractions, respectively. The relative hydrophobicity of the aggregated material is higher than would be expected on the basis of the degree of modification. Most likely the oligomeric structure has a higher binding capacity for ANSA, compared to the monomeric material. The relative hydrophobicity of the monomeric fraction is comparable to that of β-lactoglobulin using the same technique (data not shown).

Adsorption Kinetics. The adsorption kinetics for native ovalbumin and the selected caprylated sample described above were studied by measuring the increase of surface pressure (Π) and surface load (Γ) in time at the (cleaned) air–water interface of a protein solution (see parts A and B, respectively, of Figure 4). For native ovalbumin Γ starts to increase at $t = 0$ s, while during the first 600 s no significant increase of Π is measured. The end point of this lag phase (or lag time) in the $\Pi\angle t$ curve is reached when the surface load exceeds the so-called "overlap concentration". For a wide range of proteins this overlap concentration (or critical surface load, Γ_c) was found[32,33] to be in the range of 0.5–1.5 mg/m^2. As can be

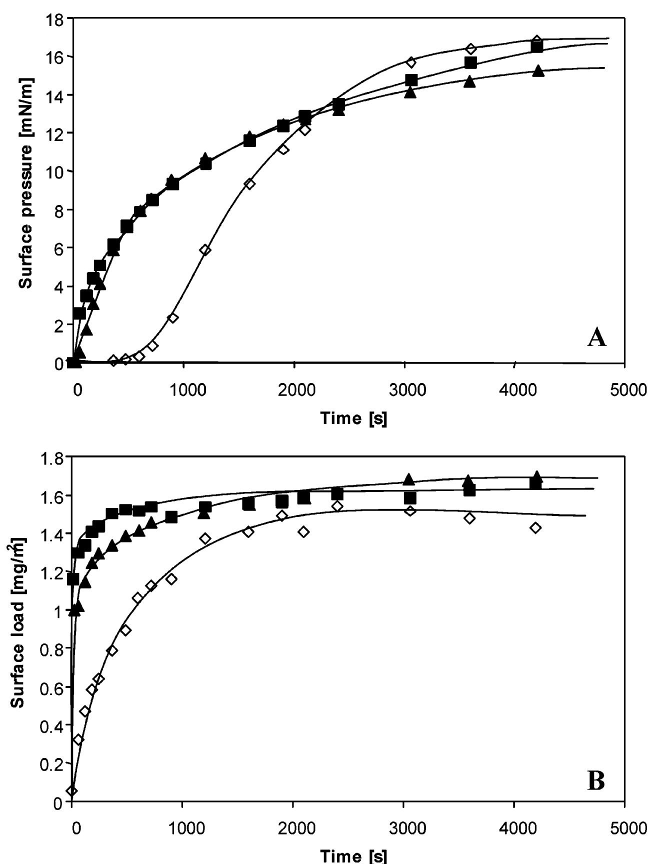

Figure 4. Surface load (A) and surface pressure (B) vs time for native (◇), monomeric caprylated (■), and aggregated caprylated (▲) ovalbumin, 0.1 mg/mL in 10 mM phosphate (pH 7.0). Measurements were taken every 20 s. Markers are shown to give an indication of the experimental error.

(32) Razumovsky, L.; Damodaran, S. Surface Activity-Compressibility Relationship of Proteins at the Air-Water Interface. *Langmuir* **1999**, *15*, 1392–1399.

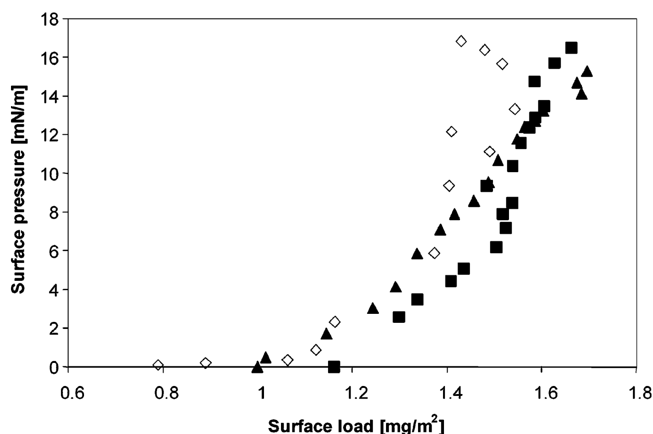

Figure 5. Surface pressure vs surface load curves (equation of state) of native (◇), monomeric caprylated (■), and aggregated caprylated (▲) ovalbumin, 0.1 mg/mL in 10 mM phosphate (pH 7.0) (markers shown correspond to the data points shown in Figure 4).

seen, for native ovalbumin Γ_c is approximately 1.1 mg/m^2. After the lag phase a decrease in the rate of adsorption is found, while the surface pressure increases rapidly. The decrease in the rate of adsorption is attributed to the amount of work that is required for a protein to "clear" enough surface area to adsorb.[2,7] The sharp increase in surface pressure in this region is the result of the interactions between the adsorbed proteins. After approximately 2000 s the surface load has reached its equilibrium value of 1.5 mg/m^2, while the surface pressure still increases between 2000 and 3000 s from 10 to 17 mN/m. This further increase in surface pressure at constant surface load is not yet fully understood, but is related to changes in the intermolecular interactions, possibly due to conformational rearrangements of the adsorbed proteins.[4,10]

For caprylated proteins a drastic change in adsorption behavior is observed. At $t = 0$ s, the surface load is already higher than Γ_c found for native ovalbumin (i.e., >1.1 mg/m^2). Thus, the proteins adsorb so fast that in the time between the cleaning of the surface and the start of the ellipsometric measurements (about 40 s) the same amount of protein adsorbed at the surface as in 600 s for native ovalbumin. The faster rate of adsorption of the caprylated ovalbumin is also demonstrated by the absence of the lag time in the $\Pi \angle t$ curve. At 1000 s, Γ has reached its equilibrium value, also at 1.5 mg/m^2, while the surface pressure continues to increase, from 10 mN/m at 1000 s to 17 mN/m at 4500 s, comparable to that of the native protein.

The aggregated fraction showed results that are comparable to those of the monomeric fraction, although the rate of adsorption is slightly slower. When the results from ellipsometry and surface tensiometry are combined, a $\Pi \angle \Gamma$ curve can be drawn. This relation between surface load and surface pressure is also referred to as the "equation of state" of the protein and provides information on interactions between adsorbed molecules. In Figure 5 the results for native and both modified fractions are shown. The surface pressure only starts to increase at a surface load of 1.1 mg/m^2 for all samples. Within the margin of error of the experiments all samples show a similar relation between Π and Γ. From these data it was concluded that the chemical modification did not result in major changes in the interactions between adsorbed protein molecules. Further experiments to gain more insight into the role of molecular properties of proteins in the surface equation of state are in progress and will be submitted for publication soon.

4. Discussion

In this study, capric acid was covalently bound to ovalbumin to increase the exposed hydrophobicity of the protein and thereby affect its adsorption kinetics. The extent of acylation can be controlled by varying the ratio of activated capric acid to ovalbumin in the reaction mixture. This control over the reaction has also been demonstrated in other studies.[14,15,23] Four batches were prepared with increasing amounts of coupled capryl chains. Even when up to 12 of the available 20 lysine groups were modified, no significant changes in secondary and tertiary structure were found. However, when more than three capryl chains were coupled to the protein, it formed aggregates. This aggregation has been reported to depend on the number of fatty acids per protein and the length of the fatty acid chains.[12,15,17,18] One batch of modified ovalbumin was fractionated in a monomeric fraction and an aggregated fraction. The (average) degrees of modification were 4 and 7 and the increases in exposed hydrophobicity were 1.4 and 6 for the monomeric and aggregated fractions, respectively. These fractions, having a similar secondary and tertiary fold, were used in the adsorption kinetics experiments.

To relate the influence of the increased exposed hydrophobicity directly to the adsorption kinetics, the adsorption kinetics of native ovalbumin was studied and compared to those of the monomeric and aggregated caprylated proteins. According to Ward and Tordai,[34] the adsorption of proteins to the air−water interface, in the limit $\Gamma = 0$, can be described by

$$\Gamma(t) = C_0(Dt/3.14)^{1/2} \quad (1)$$

where D is the diffusion constant of the protein, C_0 the bulk concentration, and t the time. More recently, models have been proposed that allow for multiple adsorption states,[35] or for the charge and unfolding of the adsorbing proteins.[36] However, here we focus on the initial stages of adsorption where the adsorbed molecules have no interactions ($\Gamma \ll 1$ mg/m^2, $\Pi \ll 1$ mN/m), so the Ward and Tordai equation should be a good approximation.

If the surface load is then plotted against the square root of time, the (apparent) diffusion constant of the protein can be calculated. When this is done for the results for native ovalbumin (shown in Figure 4), $D = 1 \times 10^{-13}$ m^2/s is found, while the actual diffusion constant of ovalbumin in the bulk solution is 7×10^{-10} m^2/s. In other words, the adsorption of ovalbumin proceeds at a much lower rate than would be expected on the basis of the diffusion rate of the protein. This indicates the existence of an energy barrier for adsorption even in the early stages of the adsorption process, contrary to the consensus of barrier-free adsorption of proteins.[4−6] However, Damodaran et al.[9] reported a similar effect for native BSA. They found that partial unfolding of the protein increased the apparent diffusion constant, indicating suboptimal adsorption of the native protein, but could not identify the underlying

(33) vanAken, G. A.; Merks, M. T. E. Adsorption of soluble proteins to dilating surfaces. *Colloids Surf., A* **1996**, *114*, 221−226.

(34) Ward, A. F. H.; Tordai, L. Time-dependence on boundary tensions of solutions. I: The role of diffusion in time-effects. *J. Chem. Phys.* **1946**, *14*, 683−696.

(35) Makievski, A. V.; et al. Adsorption of proteins at the liquid/air interface. *J. Phys. Chem. B* **1998**, *102*, 417−425

(36) Miller, R.; et al. Kinetics of adsorption of globular proteins at liquid/fluid interfaces. *Colloids Surf., A* **2001**, *183*, 381−390.

Table 2. Apparent Diffusion Constant and Calculated Energy Barrier for Adsorption of Native and Caprylated Ovalbumin

	D^* (m^2/s)	ϵ_a/kT
native	1×10^{-13}	4
monomeric	6×10^{-11}	0
aggregated	1×10^{-11}	1

mechanism. To account for the energy barrier to adsorption, Ravera et al.[37] proposed a model where the diffusion constant in (1) is replaced with a renormalized diffusion constant (D^*), which is defined as

$$D^* = De^{-\epsilon a/kT} \tag{2}$$

where ϵ_a is the activation energy for adsorption, k the Boltzmann constant, and T the absolute temperature. Using this equation, the energy barrier for the adsorption of native and caprylated ovalbumin to the air−water interface was calculated; the results are presented in Table 2. For native ovalbumin an activation energy for adsorption of 4 kT (or 10 kJ/mol) is found. A possible explanation is that the native protein needs a small conformational change before it can expose enough hydrophobic groups to the interface to adsorb. For comparison, the free energy of total unfolding of native ovalbumin is about 27 kJ/mol.[38] The monomeric lipophilized material would not need this conformational change, since it already has enough exposed hydrophobicity on the protein surface, thus resulting in a negligible value for the activation energy. The apparent diffusion constant of the momeric lipophilized material was calculated with a correction for the 40 s time period between the cleaning of the interface and the first ellipsometric data-point. And indeed a negligible value for the activation energy was found (see Table 2). For the aggregated species the bulk diffusion constant was calculated on the basis of the assumption of a spherical shape of the aggregate. Using this value (0.36×10^{-11} m^2/s), the activation energy of 1 kT was calculated. Deviations from the spherical shape will further decrease the estimate of the activation energy.

5. Conclusions

Protein exposed hydrophobicity has often been related to the kinetics of adsorption to the air−water interface. Since it is the commonly accepted view that no energy barrier for protein adsorption to "empty" surfaces can exist, hydrophobicity was said to play a role only in the latter part of adsorption, i.e., after filling of the monolayer and the subsequent development of an energy barrier. Using chemical modification, a monomeric variant of ovalbumin was obtained with an increased exposed hydrophobicity, without changes in secondary or tertiary structure. The results presented in this work suggest that the assumption of barrier-free or purely diffusion limited adsorption of proteins (at low surface load) does not hold for all cases. On the basis of these results, it is hypothesized that the energy barrier for initial adsorption of proteins is present in all cases. The height of this barrier depends on the amount of hydrophobic groups at the surface of the protein. The hydrophobic exposure on adsorption might be further increased by small conformational changes of the protein, resulting in exposure of previously buried hydrophobic groups. The energy required for such conformational changes is a part of the energy barrier for adsorption and is related to the protein structural stability.

These observations might provide new opportunities, e.g., to control foamability of food systems. Preliminary experiments demonstrated already that ovalbumin, normally a poor-foam-forming protein, upon introduction of 3−4 capryl chains on the protein surface appeared to possess reasonable foam-forming properties (results not shown). Alternatively, introduction of groups that could shield the hydrophobic exposure of ingredients might prevent foam formation.

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(37) Ravera, F.; Liggieri, L.; Steinchen, A. Sorption Kinetics Considered as a Renormalized Diffusion Process. *J. Colloid Interface Sci.* **1993**, *156*, 109−116.

(38) Kosters, H.; Broersen, K. B.; de Groot, J.; Simons, J. W. H. A.; Wierenga, P. A.; de Jongh, H. H. J. Chemical modification as a tool to generate ovalbumin variants with controlled stability, *Biotechnol. Bioeng.*, in press.