

A Kinetic Study on the Adsorption of Compact, Water-soluble Proteins onto Aqueous Surfaces

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Two compact sized globular proteins, β -lactoglobulin and α -lactalbumin were kinetically characterized at the aqueous solution surface with the measurement of surface pressure (π) and surface concentration (Γ) via a radiotracer method. The adsorption kinetics was of diffusion control at early times, the rates of increase of π and Γ being lower at longer times due to growing energy barrier. At low concentrations, an apparent time lag was observed in the evolution of π for β -lactoglobulin but not for α -lactalbumin which was shown to be due to the non-linear nature of the π - Γ relationship for the former. The area per molecule of an adsorbed β -lactoglobulin during adsorption was smaller than that for spread monolayer since β -lactoglobulin was not fully unfolded during the adsorption. For α -lactalbumin, however, no such difference in the molecular areas for adsorbed and spread monolayer was observed indicating thereby that α -lactalbumin unfolded much more rapidly (has looser tertiary structure) than β -lactoglobulin. Surface excess concentrations of α -lactalbumin was found to evolve in two steps possibly due to the change in the orientation of the adsorbed protein from a side-on to an end-on orientation.

Introduction

Proteins are known to adsorb spontaneously at gas-liquid interfaces due to their amphiphilic nature. This thermodynamically favorable process has been used in many industrial applications such as emulsions, foams and thin films with their functionality. Protein adsorption has been reported to be diffusion controlled usually for short times.^{1,2} At longer times, however, it has been shown that an adsorbing protein molecule has to overcome surface pressure as well as electrostatic energy barriers in order to anchor itself at the interface.^{3,4} Experimental observations of adsorption dynamics of succinylated β -lactoglobulin with different electrical charges have been made to demonstrate the effect of charge on energy barrier.⁵ More recent observations of surface tension dynamics of human serum albumin at air-water interface indicate that the energy barrier to adsorption may be proportional to the surface concentration.⁶ Wusneck *et al.* attribute rather high diffusion coefficients for the adsorption of β -lactoglobulin and β -casein at air-water interface to the accelerating effect of the rearrangement of adsorbed protein segments within the adsorbed layer.⁷ Hunter *et al.* explained their experimental data of adsorption isotherm and adsorption kinetics of lysozyme at the air-water interface to the change in the conformation of the molecule from side-on to end-on at higher concentrations.⁸ Upon adsorption at an interface, protein molecules are apt to unfold. The extent of unfolding of globular proteins depends on the surface pressure. The timescale of rearrangement of adsorbed protein molecule is found to be much smaller for flexible random coil proteins than globular proteins.⁹⁻¹¹

In previous work,¹² we have investigated the kinetics of bovine serum albumin (BSA) of different surface hydrophobicities in terms of both the surface pressure and surface concentration and have shown that the area occupied by BSA during adsorption is smaller than that for a spread monolayer, indicating that the protein is not completely unfolded. The extent of unfolding was also shown to depend on the conformational stability of BSA. The purpose of this paper is to elucidate the evolution of surface pressure and surface concentration during adsorption of β -lactoglobulin and α -lactalbumin at the air-water interface, which are different from BSA in structure, size and other physicochemical properties.

Materials and Methods

Materials

Isotopes of ¹⁴C-formaldehyde (H¹⁴CHO), ¹⁴C-carboxystearic acid and ¹⁴C sodium acetate (purchased from Sigma Chemicals) were used for radiolabelling. β -lactoglobulin (Prod. N L0130, lot Number 114H7055) and α -lactalbumin (Type III, Prod. Nb L6010, lot Number 128F8140), purchased from Sigma Chemicals, were treated with charcoal (charcoal : protein = 6 : 1) so as to eliminate any possible low-molecular weight surface active impurities.¹³ Phosphate buffer (0.01 M, pH 7.4, 0.09% NaCl) was prepared from highly purified de-ionized water in all the experiments.

A computer-controlled Langmuir minitrough (KSV, Finland) with a Whilhelmy plate was used for adsorption kinetics and spreading experiments as described elsewhere.^{14,15} β -lactoglobulin and α -lactalbumin were radiolabelled with ¹⁴C

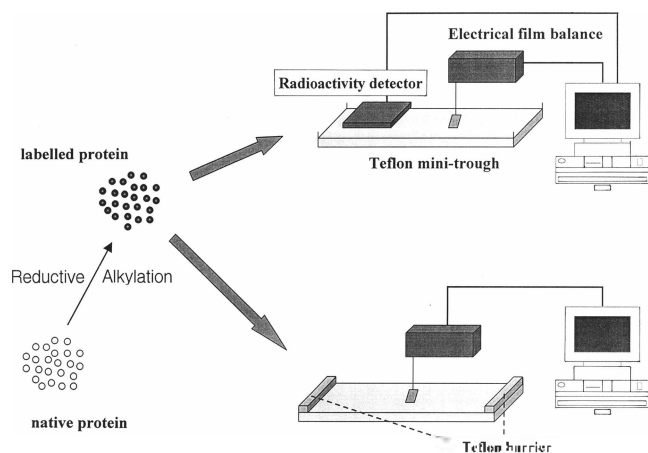


Figure 1. Schematic of experimental setup in which two main operations (adsorption and spreading/compression) are performed.

in order to enable direct measurement of adsorbed protein concentration at the air-water interface (see details in "Methods"). A gas proportional detector (Ludlum instrument model 120 with a 2×2 thin mylar window) with a digital scale counter (Ludlum instrument model 520) was used for detecting radioactivity.

Schematic of experimental setup and two important operations are shown in Figure 1.

Methods

Labelling of proteins. Protein was radiolabelled by reaction with $^{14}\text{HCHO}$ in the presence of 0.1 M NaCNBH_3 .^{12,14} Thirty milligrams of protein powder was dissolved in 6 mL of 0.05 M phosphate buffer (pH 7) and addition of 750 μL of 0.1 M NaCNBH_3 to the protein solution was followed. Six milliliters (equivalent to 102 μCi) of $^{14}\text{HCHO}$ stock solution was then mixed with the above protein solution and reaction was allowed to proceed for 2 h under ambient condition. After the reaction, the mixture was immediately put in a dialysis membrane tubing (SpectraPOR from Spectrum Inc.) with MW cut-off of 6000-8000 and was dialyzed for 20 h at 4 $^\circ\text{C}$ for complete removal of unreacted species. Protein concentration of the final solution was determined using BCA assay.¹⁶ Radioactivity of the final protein samples was measured using a scintillation counter (Packard model Tri-carb 4000) calibrated with known ^{14}C sample in counts per minute (CPM). Each protein sample (20-100 μL) was mixed with 6 ml of Ecolume cocktail solution and the CPM of the mixture was measured with the scintillation counter and converted to μCi using a calibration curve (CPM versus μCi of known samples).

Adsorption from solution. The Langmuir minitrough was first filled with a buffer solution without any protein. The air-water interface was then carefully aspirated to remove surface impurities and the surface pressure was adjusted to zero. Then, a quiescent solution of ^{14}C labelled protein in phosphate buffer was placed into the Langmuir minitrough. The Ludlum detector was immediately positioned and both of the surface pressure and CPM of β -radiations were monitored by an automatic data acquisition system. Adsorption experiments were normally durated for 20 h. In order to con-

vert CPM to the surface concentration, the Ludlum gas proportional detector was calibrated with radioactive samples of known surface and bulk concentrations as reported in elsewhere.¹⁷ Different amounts of radiolabelled proteins were spread on the air-water interface using Trumits method.¹⁸ The surface of the air-water interface was compressed in stages and a calibration curve relating CPM versus surface concentration was constructed. A different calibration curve was constructed for each protein studied.

Surface Pressure-Area isotherms for spread monolayer

Protein monolayers were spread using Trumits method. A 50 μL aliquot of protein solution of 0.0247 wt% concentration was dripped from the top of a glass rod (5 mm diameter, 5 cm height) positioned above the air-water interface so that the solution spread uniformly on the top of the interface.^{14,15} It has been shown by Cho *et al.* that the loss of protein due to desorption from the air-water interface is negligible.¹⁹ The spread monolayer was allowed to equilibrate for 30 min and was then compressed by moving the two teflon barriers. The surface pressure was recorded at different areas and the surface concentration Γ of the protein was converted from the relation, *i.e.* $\Gamma=I/A$ under the "no loss" assumption.

Results and Discussion

The evolution of surface pressure (π) and surface concentration (Γ) for different bulk concentrations of β -lactoglobulin and α -lactalbumin are given in Figures 2 and 3.

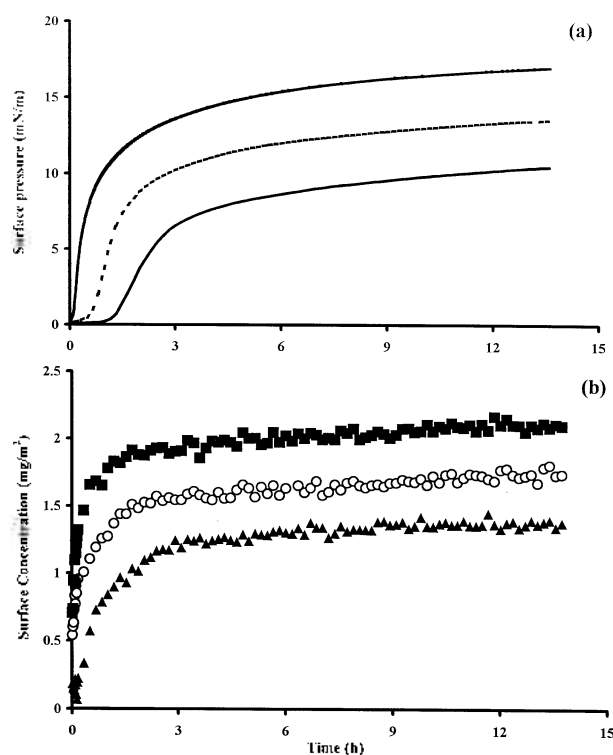


Figure 2. Kinetic evolution of ^{14}C - β -lactoglobulin for different bulk concentrations in terms of (a) $\pi(t)$. (—) 0.5 g/m^3 , (---) 1.0 g/m^3 , (· · ·) 2.0 g/m^3 , and (b) $\Gamma(t)$. \blacktriangle 0.5 g/m^3 , \circ 1.0 g/m^3 , \blacksquare 2.0 g/m^3 .

respectively. The rates of increase in π and Γ ($d\pi/dt$, $d\Gamma/dt$) were higher for the higher protein concentrations, especially at short times. For the most dilute solution of β -lactoglobulin, an apparent lag time was observed for π , but not for Γ (see Figure 2). At 0.5 g/m³ of bulk concentration, the induction period, during which π was negligible ($\pi < 1$ mN/m) lasted up to 85 min after which the surface pressure was found to increase rapidly. Induction time was found to decrease as the bulk concentration was increased. No significant lag time was observed for Γ (Figure 2b). In the case of α -lactalbumin, lag time was not observed either for π , or for Γ (Figure 3). While surface pressure increased progressively, the rate of increase in surface pressure was found to be lower than that for β -lactoglobulin. The rate of increase in surface concentration was found to be higher for α -lactalbumin than for β -lactoglobulin for bulk concentrations of 0.5 and 1.0 g/m³.

Such an induction period for the evolution of π has been reported in many past works.^{7,10,15,20-21} According to De Feijter and Benjamins,²² π remains extremely low until a sufficient surface concentration has been built up because of the non-linear nature of the surface equation of state. Experimental observation of such an induction period for bovine serum albumin has been shown to be due to the fact that π is insignificant whenever Γ is below a certain critical value (Γ_{crit}) because of the non-linear nature of the adsorption isotherm.¹⁵ Wei *et al.* measured the kinetics of surface tension for five model proteins and found that the rate at which the surface tension decreases was correlated with the conformational stability of the proteins.²³ They attributed this induction period to the time needed for unfolding of the protein molecule upon adsorption at the air-water interface. That time would depend upon conformational stability of protein molecules. Data of $\pi(t)$ and $\Gamma(t)$ can construct an "isotherm", $\pi(\Gamma)$ during adsorption (Figure 4). From these curves, $\Gamma_{\pi=1}$,

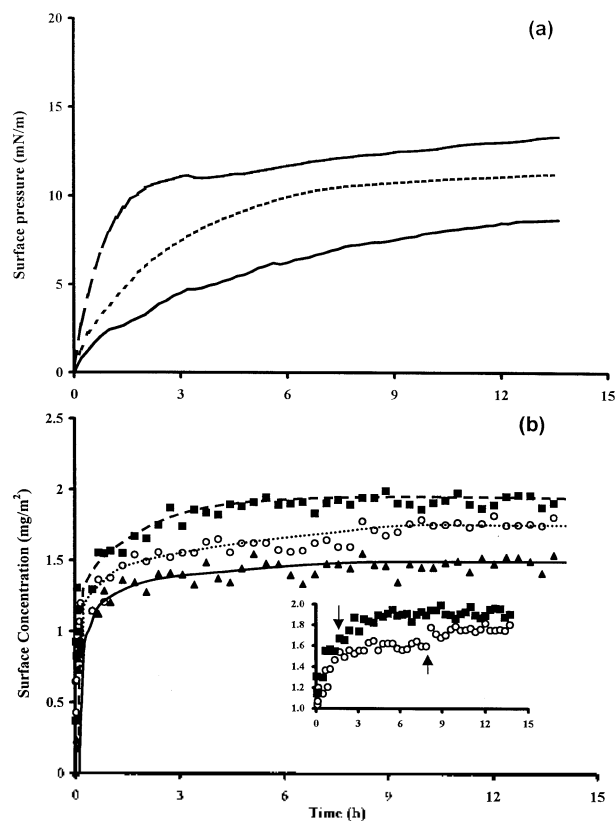


Figure 3. Kinetic evolution of ¹³C- α -lactalbumin for different bulk concentrations in terms of (a) $\pi(t)$, (—) 0.5 g/m³, (----) 1.0 g/m³, (---) 2.0 g/m³, and (b) $\Gamma(t)$, \blacktriangle 0.5 g/m³, \circ 1.0 g/m³, \blacksquare 2.0 g/m³. Arrows on the captured figure point to "inflection".

the protein surface concentration needed for π to be equal to 1 mN/m, can be determined (Table 1). From $\Gamma(t)$ and $\pi(t)$, $t_{\Gamma(\pi=1)}$, the time for which $\Gamma_{\pi=1}$ is reached can be determined.

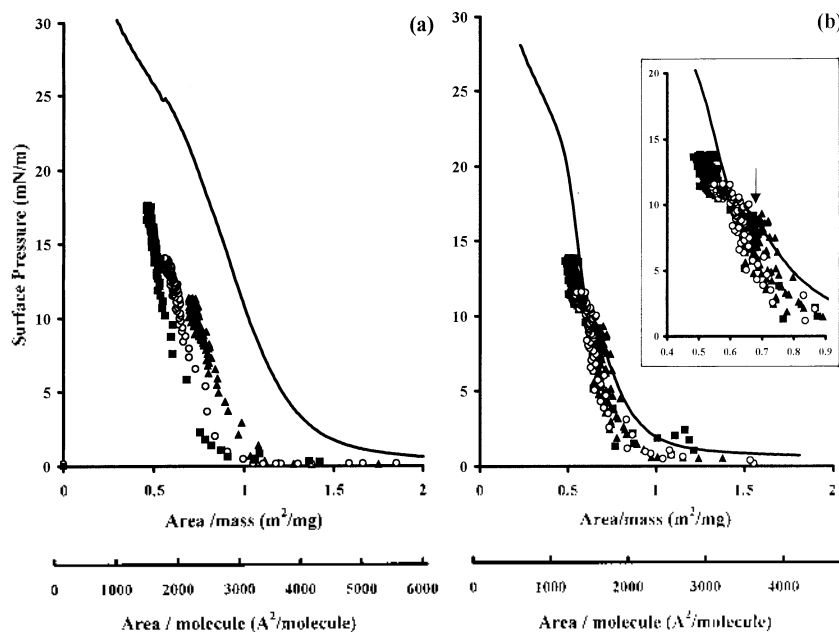


Figure 4. Comparison of dynamic $\pi(t)$ - $\Gamma(t)$ plots (symbols) to spread monolayer π - Γ isotherm (solid line) for (a) β -lactoglobulin and (b) α -lactalbumin, \blacktriangle 0.5 g/m³, \circ 1.0 g/m³, \blacksquare 2.0 g/m³.

Table 1. Mutual relationship between Γ and time when $\pi=1$ mN/m at different bulk concentrations

	Bulk concentration (g/m ³)	$\Gamma_{\pi=1}$ (mg/m ²)	$t_{\Gamma(\pi=1)}$ (h)	$t_{\pi=1}$ (h)
α -lactalbumin	0.5	1.07	0.34	0.31
	1.0	0.92	0.10	0.15
	2.0	0.98	0.03	0.053
β -lactoglobulin	0.5	0.92	1.50	1.50
	1.0	1.10	0.51	0.65
	2.0	1.14	0.12	0.12

Table 2. Values of ΔA_1 and ΔA_2 , so-called surface clearing areas obtained at the two stages of adsorption (refer to Figure 5)

	Bulk concentration (g/m ³)	ΔA_1 (Å ²)	ΔA_2 (Å ²)	$\Delta A_2/\Delta A_1$
α -lactalbumin	0.5	270	N/A	–
	1.0	171	N/A	–
	2.0	95	852	9.0
β -lactoglobulin	0.5	55	332	6.0
	1.0	59	259	4.4
	2.0	64	279	4.4

It can be observed that for both proteins studied and for every bulk concentration investigated, the surface concentration needed for $\pi=1$ mN/m is fairly close ($0.94 < \Gamma_{\pi=1} < 1.10$ mg/m²) but the time at which this critical surface concentration is reached depends both on the bulk concentration as well as on the type of protein. Moreover, $t_{\Gamma(\pi=1)}$ was found to be similar to the time at which π reached 1 mN/m ($t_{\pi=1}$) i.e. the induction period as determined from the $\pi(t)$ curve. Consequently, it appears that the observed induction period in $\pi(t)$ is due to the non-linear nature of the $\pi(\Gamma)$ relationship during adsorption.

A plot of Γ versus \sqrt{t} (not shown here) showed that adsorption was diffusion controlled only in the first 10 minutes for both proteins. At longer times, the plot was non-linear thus indicating the presence of energy barriers against adsorption. The diffusion coefficients inferred from the plots (see Table 2) were found to be higher at lower concentrations, being consistent with the earlier observations reported in the literature.⁷

The induction period observed for β -lactoglobulin could be due to the fact that it needs more time for β -lactoglobulin to reach the surface concentration at which π starts increasing compared to that for α -lactalbumin. Based on the monomer-dimer equilibrium constant of 5.6×10^{-6} mol/l at pH 7 and 20 °C,^{24,25} it can be concluded that β -lactoglobulin is a monomer under the conditions employed in this study. The α -lactalbumin used in this work is the apo-form (it contains less than 0.3 mol Ca²⁺ per mol of protein). However, Na⁺ ions present in the buffer can bind to the two Ca²⁺ sites of α -lactalbumin leading to a native-like structure.²⁶ According to Comec *et al.*,²⁷ the near UV circular dichroism spectrum of

the native-like α -lactalbumin was found to be similar to the one of the metalloprotein²⁸ thus suggesting that the tertiary structure of the apo-protein in a phosphate buffer is similar to the tertiary structure of the Ca²⁺- α -lactalbumin. The structure of α -lactalbumin is stabilized by 4 disulfide bonds which are compared to 2 for β -lactoglobulin. However, the latter protein contains a free thiol group which can react and form new disulfide bonds leading to a reduction of its flexibility.² This is consistent with the fact that the thermal stability of α -lactalbumin is lower than that of β -lactoglobulin. The onset of denaturation occurs at 59–62 °C for α -lactalbumin as compared to 76–82 °C for β -lactoglobulin.³¹

Steady state surface concentrations were found to be comparable for both proteins whereas steady state surface pressures were found to be higher for β -lactoglobulin. This suggests that the adsorbed molecules of β -lactoglobulin exerts more effect on the surface pressure than the adsorbed α -lactalbumin at higher Γ (smaller areas per molecule) even though the effect was just the opposite for much larger (>1 m²/mg) molecular areas. Corredig and Dalgleish reported that denaturation of α -lactalbumin upon adsorption at the oil-water interface was reversible suggesting that no breakdown in disulfide bonds occurred upon adsorption.³¹ On the other hand, β -lactoglobulin showed the highest degree of denaturation upon adsorption and the conformational changes were irreversible.

At intermediate times, the surface concentration of α -lactalbumin reached a plateau, or inflection point, after about 4 hours for a bulk concentration of 1.0 g/m³ and after 2 h for 2.0 g/m³ (see inset of Figure 3b). The plateau was more pronounced and was extended over a longer period of time for 1.0 g/m³. In both of the cases, the plateau corresponded to a surface concentration of 1.5–1.6 mg/m². No plateau was observed at a bulk concentration of 0.5 g/m³ since the steady state Γ was less than 1.5–1.6 mg/m². At longer times, surface concentration increased again up to steady state values of about 1.75 mg/m² for a bulk concentration of 1.0 g/m³ and 1.9 mg/m² for a bulk concentration of 2.0 g/m³. Existence of such a plateau has already been reported for adsorption of α -lactalbumin at solid-water interface.³² It was suggested that the orientation of the adsorbed proteins change from a side-on to an end-on as the surface concentration increased. α -lactalbumin has the ability to fully renature after thermal denaturation. Under these conditions, it is conceivable that when rapid adsorption was followed by a partial denaturation, the protein adopted more energetically favorable states, even in a crowded interface. Similar change in the orientation was also suggested by Hunter *et al.* for adsorption of lysozyme at air-water interface. No plateau was observed in the case of β -lactoglobulin.⁸ This is consistent with an irreversible conformational change upon adsorption at the interface as observed by Corredig and Dalgleish.³¹

From $\pi(t)$ and $\Gamma(t)$ curves, the dynamics π - Γ plot can be constructed. As can be seen from Figure 4a, the experimental π - Γ plots for different concentrations of β -lactoglobulin do not fall into a single curve. Dynamic π - Γ data for β -lactoglobulin are compared with the π - Γ isotherm obtained by

spread monolayer using Truitt's method. It can be seen that the surface pressure during adsorption is found to be smaller than that given by the spread monolayer isotherm for the same surface concentration. In other words, for the same surface pressure, the area occupied by the protein molecule during adsorption (inversely proportional to Γ) is smaller than that for spread monolayer. Consequently, the protein molecules in the spread monolayer are more unfolded. Mitchell *et al.* compared spread monolayer isotherms from native and denatured milk globular proteins and observed that the spread monolayer isotherms were independent of the structural state of the protein.³³ It was, therefore, concluded that all the molecules in a dilute spread monolayer are in an extensively unfolded configuration. On the other hand, isotherms constructed by spreading increasing amounts of protein were strongly dependent on the structure of the molecule. As pointed out by these authors, the essential difference between the formation of a spread monolayer and an adsorbed film is that in the spread film, all the molecules have initially entered the surface when the surface pressure is zero, while in an adsorbed film, this is only true for the first molecules that are adsorbed. β -lactoglobulin molecules that arrive at the interface later will have to adsorb against the surface pressure developed by previously adsorbed molecules. As the bulk concentration is increased, the area occupied by the β -lactoglobulin molecules upon adsorption is decreased, which suggests that at higher bulk concentrations, protein molecules will have less room at the interface and are thus not allowed to unfold completely.

On the other hand, dynamic π - Γ plots for α -lactalbumin at different concentrations fall into a single curve and agree fairly well with the π - Γ isotherm obtained by spread monolayer (Figure 4b). Both dynamic π - Γ plots as well as the spread monolayer isotherm exhibit an inflection point at $\pi=11$ mN/m which is in good agreement with published results.⁷ Such an inflection point has also been observed for homologous lysozyme (at $\pi=8$ mN/m),⁷ ovalbumin³² but not for BSA.^{7,8} The inflection point is believed to mark the point where loops and tails become predominant at the interface and where adsorbing globular proteins are prevented from unfolding by the pressure of the molecules already in the film.^{7,34} The fact that this inflection point was not observed for heat-denatured lysozyme, reduced α -lactalbumin⁷ suggests that α -lactalbumin does not completely unfold and that some tertiary structure elements are retained in the monolayer. In contrast, the absence of an inflection point in the dynamic and spread monolayer isotherms of β -lactoglobulin implies that no native β -lactoglobulin molecule is retained in the monolayer.

The area needed to be cleared by an adsorbing protein in order to anchor at the interface can be determined from the plot of $\ln(d\Gamma/dt)$ versus π .³ Plots for β -lactoglobulin exhibit two different regions of different slopes corresponding to two average interfacial areas, one ΔA_1 for short times and a second ΔA_2 for long times. The values are reported in Table 2. The average ΔA_1 values at short times were found to be in the range of 55-63 \AA^2 whereas the average ΔA_2 were around

259-332 \AA^2 . Plots obtained from α -lactalbumin exhibited only one region at protein bulk concentrations of 0.5 and 1.0 g/m³ with ΔA_2 value of 171-270 \AA^2 . The absence of the first region suggests that α -lactalbumin unfolds more rapidly upon adsorption than β -lactoglobulin. ΔA_2 values obtained for α -lactalbumin were lower than those for β -lactoglobulin, thus indicating that unfolding of the former is more limited.

Conclusions

Dynamics of adsorption of ¹⁴C-radiolabelled β -lactoglobulin and α -lactalbumin at the air-water interface was investigated. The rate of increase as well as the steady state value of π and Γ were found to be higher for higher bulk concentrations, especially at short times. At low concentrations, an apparent time lag, more pronounced for π than for Γ , was observed for β -lactoglobulin but not for α -lactalbumin. This behavior is shown to be due to the non-linear nature of π - Γ relationship for β -lactoglobulin. The area per molecule of an adsorbed β -lactoglobulin molecule during adsorption was smaller than that for the spread monolayer thus indicating that β -lactoglobulin was less unfolded during adsorption. This was not the case for α -lactalbumin for which no difference was observed in the area per molecule between adsorbed and spread protein layers. Two different ΔA values corresponding to the area that need to be cleared at short times (ΔA_1) and at longer times (ΔA_2) were determined for β -lactoglobulin. Only one ΔA was found for α -lactalbumin suggesting that α -lactalbumin unfolded more rapidly than β -lactoglobulin but to a lesser extent. An inflection point was observed in both the dynamic π - Γ relationship and the spread monolayer isotherm for α -lactalbumin suggesting that some tertiary structure remained in the adsorbed molecule. This was not true for β -lactoglobulin.

It was concluded that β -lactoglobulin was more denatured upon adsorption than α -lactalbumin. Evolution of the surface concentration of α -lactalbumin with time was found to occur in two steps. It is believed to be due to a change in the orientation of adsorbed proteins from a side-on to an end-on orientation as the interface becomes more crowded.

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