β-Casein Adsorption at Liquid Interfaces: Theory and Experiment

Julia Maldonado-Valderrama,[†] Alberto Martín-Molina,[‡] Maria José Gálvez-Ruiz,[†] Antonio Martín-Rodríguez,[†] and Miguel Ángel Cabrerizo-Vílchez*[,†]

Biocolloid and Fluid Physics Group, Department of Applied Physics, Faculty of Science, University of Granada, Fuentenueva, s/n, 18071 Granada, Spain, and Laboratoire de Physique Statistique de l'Ecole Normale Supérieure associée au CNRS et aux universités Paris VI et Paris VII, 24 rue Lhomond, 75231 Paris Cedex 05, France

Received: April 13, 2004; In Final Form: June 21, 2004

A procedure of analysis of protein adsorption isotherms at liquid interfaces is proposed in terms of the steady state $\pi-c$ curves and a thermodynamic model. Such practice is applied in the study of β-casein, a random coil protein that seems to be very dependent on the nonpolar phase onto which it is adsorbed. First, differences in the interfacial behavior of the protein are drawn from the experimental data recorded at the air−water and tetradecane−water interfaces. Second, the application of the model suggests that the interface significantly affects the interfacial denaturation undertaken by the protein. More specifically, it provides the actual interfacial area occupied by the protein at both interfaces indicating a further unfolded state attained by β-casein at the tetradecane−water interface. Finally, the procedure is also applied to literature data to clarify the apparent discrepancies found on the adsorption of β-casein at different liquid interfaces.

I. Introduction

Although considerable effort has been done in the last decades with the aim of comprehending how proteins adsorb onto interfaces, the mechanism of protein adsorption and the structure of their interfacial layers are still under investigation.[1−3] Indeed, this deficit is more noticeable for the case of protein adsorption at liquid interfaces where the problem, nowadays, remains unsolved.[4] Oil interfaces constitute a wide scientific field with important applications in various different topics going from food technology (formation and stability of emulsions)[5] to their use as simple models of the water−membrane interface.[6] Experimentally, the difficulties to perform studies at oil interfaces results in a certain lack of results. In this respect, it seems to be assumed that an oil interface allows more penetration of the unravelled proteins into the nonpolar phase.[7−10] This experimental feature agrees with theoretical findings[3,11] and even with recent molecular simulation results.[2,6] However, details on the interfacial configuration adopted by proteins at oil interfaces are still unclear. With regard to this scarcity of information, the primary objective of this work is to show experimental results of adsorption of β-casein at the air−water and oil−water interfaces. Specifically, a pendant drop Langmuir-type film balance, based on axisymmetric drop shape analysis (ADSA), has been employed for studying the adsorption of this protein at the air−water and the tetradecane−water interfaces. This device provides analysis of the different behavior by recording the interfacial tensions of protein solutions in relatively simple experiments. Nevertheless, the information derived from these experimental curves appears insufficient to obtain the composition of the respective interfacial layers. At this point emerges a second objective of this work; besides comparing the results at both interfaces, we analyze them within a thermodynamic model of protein adsorption developed by Fainerman et al.[12−14] This model assumes the unfolding process undertaken by the protein upon adsorption by means of a generalization of the concept formulated by Joos and Serrien[15] in which proteins coexist at the interface in various states with different partial molar area. The validity of this theory has been proven with several proteins at the air−water interface.[16] However, its comparison with experimental data at the oil−water interface has not been made up till now.

In relation to the experimental systems employed, β-casein appears as a well-known random coil protein very sensitive to the nonpolar phase onto which it is adsorbed as can be seen in the literature[3,7,9,17−19] in view of the discrepancy found in the literature with different oil phases. To shed some light on this apparent quarrel, the oil phase chosen for this work is tetradecane, and a comparison with Graham and Phillips's results (which were obtained by using the same protein but a different oil phase as well as a different experimental setup) has been made.[17] Moreover, the procedure of data analysis proposed is also applied to these results with the aim of confirming that the nature of the interface significantly affects the interfacial conformation finally adopted by the protein.

Accordingly, the paper is organized as follows. In the next section, the theory of protein adsorption developed by Fainerman et al. is briefly reviewed.[12−14] Subsequently, the samples used for this study and the experimental technique are presented. In section III, all the results are exposed in this way: (i) The experimental results of adsorption of β-casein at air−water and tetradecane−water interfaces are presented. (ii) The experimental curves are fitted by using the previously referred theory of protein adsorption. (iii) The final part of this section is dedicated to discuss the role of the nature of the interface in view of the theoretical considerations. Furthermore, the procedure is also applied to literature data to clarify the discrepancies reported

* Corresponding author. Tel: 0034 958 243211. Fax: 0034 958 243214. E-mail address: mcabre@ugr.es.
[†] University of Granada.
[‡] Laboratoire de Physique Statistique de l'Ecole Normale Supérieure associée au CNRS et aux universités Paris VI et Paris VII.

10.1021/jp048388y CCC: $27.50
Published on Web 07/31/2004

on the structural configuration attained by β-casein at different liquid interfaces. Finally, some conclusions are pointed out.

II. Theoretical Backround

The theory of protein adsorption proposed by Fainerman et al. and used in this work is described in detail in refs 12−14; however, a brief description will be given at this point. The theory develops the idea of Joos and Serrien in which proteins adsorb onto the interface with two different partial molar areas[15] to a generalization in which multiple states of the protein molecule can exist in the interfacial layer. Specifically, the partial molar area per molecule of any *i*-state differs from that of the neighboring states, $i \pm 1$, by the incremental value $\Delta\omega$, which is determined by the flexibility of the protein molecule. The range over which the partial molar area can vary is restricted to certain maximum and minimum values, ω_{max} and ω_{min}, respectively, that are also fixed by the geometric dimension of the protein molecule. Finally, $\omega_{max} = \omega_{min} + (n-1)\Delta\omega$, where n is the number of states. The composition of the interfacial layer is controlled by the interfacial pressure so that the part of molecules with a minimal interfacial area demand increases with increasing interfacial pressure. The starting point of the thermodynamic analysis performed is Butler's equation for the chemical potentials of the *i*-state of a protein molecule within the interfacial layer and in the bulk solution.[12] With these premises, one obtains the equations of state for the adsorption layer and the adsorption isotherm in terms of the activity coefficients and the surface coverage. Subsequently, the authors choose the dividing surface as defined by Lucassen-Reynders:[20,21]

$$\sum_{i=0}^{n}\Gamma_i = \frac{1}{\omega_\Sigma}; \quad \omega_\Sigma = \frac{\sum_{i=1}^{n}\Gamma_i\omega_i}{\sum_{i=1}^{n}\Gamma_i} \tag{1}$$

where Γ_i is the adsorption of any state given by

$$\Gamma_i = \Gamma_\Sigma \frac{i^\alpha \exp\left(-\dfrac{(i-1)\pi\omega_1}{RT}\right)}{\sum_{i=1}^{n} i^\alpha \exp\left(-\dfrac{i\pi\omega_1}{RT}\right)} \tag{2}$$

where $\pi = \gamma_0 - \gamma$, γ_0 and γ are the interfacial pressures of the solvent and solution, respectively, R is the gas constant, T is the absolute temperature, α is a measure of the difference in the surface activity of the various states, $\omega_1 = \omega_{min}$, and Γ_Σ is the total adsorption defined as follows $\Gamma_\Sigma = \sum_{i=1}^{n}\Gamma_i$.

The value ω_Σ represents the mean molar area defined as the weighted average over all states of protein in the interfacial layer:

$$\omega_\Sigma = \omega_1 \frac{\sum_{i=1}^{n} i^{(\alpha+1)} \exp\left(-\dfrac{i\pi\omega_1}{RT}\right)}{\sum_{i=1}^{n} i^\alpha \exp\left(-\dfrac{i\pi\omega_1}{RT}\right)} \tag{3}$$

With these expressions and introducing the equations for the activity coefficients in terms of the enthalpy and entropy of mixing[12] and the contribution of the interion interaction, which arises as the main contribution to the surface pressure,[13] one can rigorously derive and simplify the following expressions for the equation of state and the adsorption isotherm:

$$\pi = -\frac{RT}{\omega_\Sigma}[\ln(1-\Gamma_\Sigma\omega_\Sigma) - a_{el}\Gamma_\Sigma^{\,2}\omega_\Sigma^{\,2}] \tag{4}$$

and

$$b_1 c = \frac{\Gamma_1\omega_\Sigma}{(1-\Gamma_\Sigma\omega_\Sigma)^{\omega_1/\omega_\Sigma}} \tag{5}$$

where c is the bulk concentration, a_{el} is the interion interaction parameter, and b_1 is an equilibrium constant.

This set of equations describes the adsorption within the range of simultaneous increase of surface pressure and adsorption. Assuming the existence of a critical protein concentration above which the adsorption appears pressure-independent, the theory has been recently extended to this region by taking into account aggregations in 2D between proteins at the surface layer. Hence, the equation of state and the adsorption isotherm for this range of surface pressure are given by

$$\pi = -\frac{RT}{\omega_1}\left[\frac{\Gamma_c}{\Gamma_\Sigma^*}\ln(1-\Gamma_\Sigma^*\omega_1) - a_{el}\Gamma_c^{\,2}\omega_1^{\,2}\right] \tag{6}$$

and

$$b_1 c = \frac{\Gamma_\Sigma^*\omega_1}{(1-\Gamma_\Sigma^*\omega_1)} \tag{7}$$

where Γ_c is the critical adsorption of protein aggregation in the surface layer and Γ_Σ^* is that total adsorption accounting also for the aggregation in the adsorption layer. Details on this derivation can be found in ref 14.

III. Experimental Section

Materials. Lyophilized, essentially salt-free bovine milk β-casein (90+% by electrophoresis) was purchased from Sigma Chemical Co. β-casein is a model protein widely studied in the literature. It presents a random coil, asymmetric configuration and has an extremely flexible structure. It is made up of 209 amino acids, and it has a molecular weight of 23.8 kDa; the entire molecule has an average hydrophobicity of 5.58 kJ/residue.[9] It was stored at −18 °C and used without further purification. The oil phase chosen in this study is *n*-tetradecane (99+%) purchased from Aldrich, purified by chromatography resins Florisil 60−100 mesh and subsequently filtered with 0.2 μm PTFE filters. The aqueous subphase used is a phosphate-buffered saline (PBS): 13.0 mM KH_2PO_4 (purissimum, Merck), 100 mM NaCl (p.a., Merck), 54.0 mM Na_2HPO_4 (p.a., Merck) of pH 7.4. To prevent bacterial contamination, 0.5 g/L NaN_3 was added to the buffered solvent. Solutions were prepared daily and 0.054 μS Milli-Q+ purified water was used for buffer preparation and all other purposes. All experiments were performed at $T = 23$ °C. The surface tension, γ_0, of the clean interface was measured before each experiment to ensure the absence of surface active contaminants in the solution obtaining values between 72.5 and 73 mJ/m^2. Regarding the tetradecane, even after the purification process, it showed a low level of surface-active impurities. However, it has been proven that the interfacial activity of the proteins overcomes the effect of the

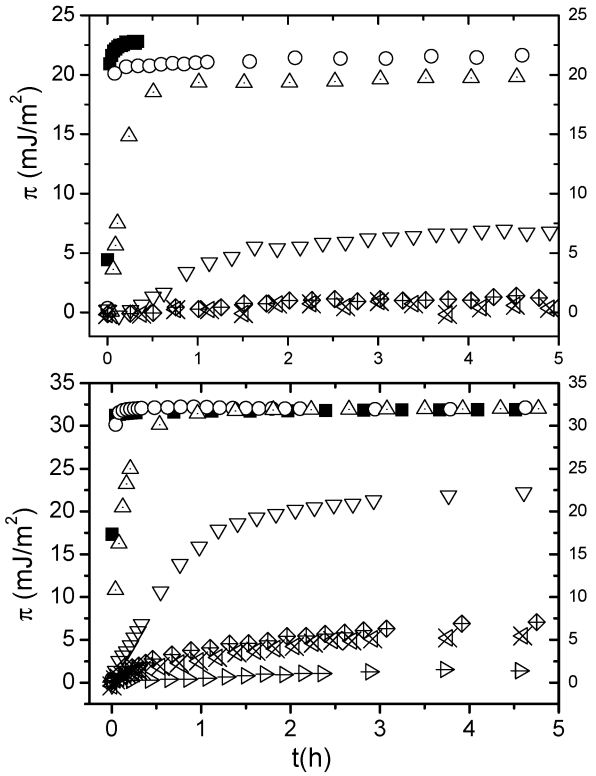

Figure 1. β-casein adsorption dynamics at the air−water (upper) and the oil−water (lower) interfaces: (■) 10^{-1} g/L; (○) 3×10^{-2} g/L; (△) 10^{-2} g/L; (▽) 3×10^{-3} g/L; (crossed rhombus) 10^{-3} g/L; (crossed left triangles) 10^{-5} g/L; (crossed right triangles) 10^{-6} g/L.

low impurities present as the interfacial tension value for the tetradecane−water interface would remain between 50 and 53 mJ/m^2 after 20 h.[8,10]

Experimental Setup. The present study was performed with a constant surface pressure penetration Langmuir balance based on axisymmetric drop shape analysis (ADSA), which is described in detail in ref 22. The image capturing, the microinjector, and the ADSA algorithm are managed by a Windows integrated program (DINATEN). The program fits experimental drop profiles, extracted from digital drop micrographs, to the Young−Laplace equation of capillarity by using ADSA and provides as outputs the drop volume, V, the interfacial tension, γ, and the interfacial area, A. The drop is immersed in a glass cuvette (Hellman) that is kept in a thermostatized cell. To obtain the liquid interface, the cited cuvette is filled with the less dense phase providing the adequate media to form the interface. The solution droplet is formed at the tip of a coaxial double capillary, connected independently to a double microinjector. The double capillary is used for creating the drop in which the adsorption is studied. The outer capillary injects protein solution at the same time as the inner one extracts enough liquid so that the area of the drop is kept constant during the measurement by injecting or extracting solution into the drop.[22] The adsorption of β-casein is hence measured by recording the change of interfacial tension at constant interfacial area, 28 and 35 mm^2 for the air−water and the oil−water interfaces, respectively.

IV. Results and Discussion

To obtain the equilibrium values with the adjacent bulk solution, suitable for the application of the theoretical model, the adsorption is recorded in terms of the interfacial tension in very large periods of time (20 h). Figure 1 shows the dynamic adsorption curves for β-casein at the air−water and oil−water interface.

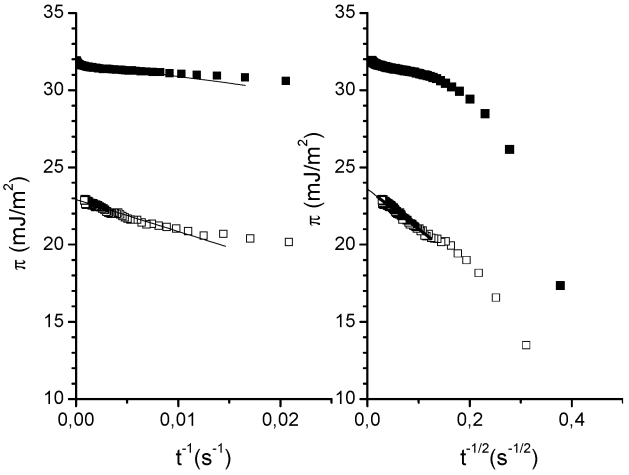

Figure 2. Extrapolation of the dynamic curves at the air−water (open symbols) and the tetradecane−water (solid symbols) interfaces.

It is worth noting how equilibrium is only rapidly attained for the most concentrated bulk solutions at both interfaces in agreement with previous studies at the air−water and the oil−water interfaces.[8,23,24] In contrast, differences between adsorption onto both interfaces arise from comparison of the respective dynamics curves. In particular, it can be seen how for each bulk concentration the rate of interfacial pressure is higher at the tetradecane−water interface. Beverung et al. find similarly higher rates for β-casein at the heptane−water interface when compared with globular proteins and relate them to the disordered structure of β-casein in the bulk.[8] Also, Segumpta et al. connect the differences in the rate of adsorption of proteins of β-casein at the triolein−water interface with respect to the air−water interface mainly to differences in the dispersion interaction between proteins and the nonpolar phases.[9] This interaction seems to be always attractive in the case of oil interfaces, and it could be responsible, therefore, for the higher rate of β-casein adsorption recorded in Figure 1 at the tetradecane−water interface. At this point, it is remarkable that in none of the works cited above a direct comparison of the dynamic curves at both interfaces is presented.

Given that evidence of the fact that the nature of the interface affects the process of protein adsorption arises from comparison of the equilibrium interfacial tension values attained by β-casein at both interfaces, let us analyze the steady-state adsorption isotherms. To this end, the equilibrium interfacial tension values have been obtained by following the procedure described in ref 25. By standards of this, the values can be estimated by the extrapolation of the derivatives $d\gamma/dt^{-1/2}$ and $d\gamma/dt^{-1}$ by considering a mixed adsorption mechanism. These two extrapolations correspond to the consideration of a diffusion-controlled adsorption mechanism and to the presence of an adsorption barrier, respectively.[25] The former approximation leads to the well-known Joos−Hansen relationship;[25] however, the values estimated from the extrapolated curves are somehow higher than those predicted by this equation. This question is addressed in detail by Wüsneck et al. in ref 26 and points out the fact that this formalism was originally developed for surfactants.[26] To account for this feature, the second extrapolation is performed to the adsorption data as described in ref 13.

As can be seen in Figure 2, the equilibrium interfacial tension values estimated by the two different extrapolation procedures are very close to each other. Moreover, due to the very long

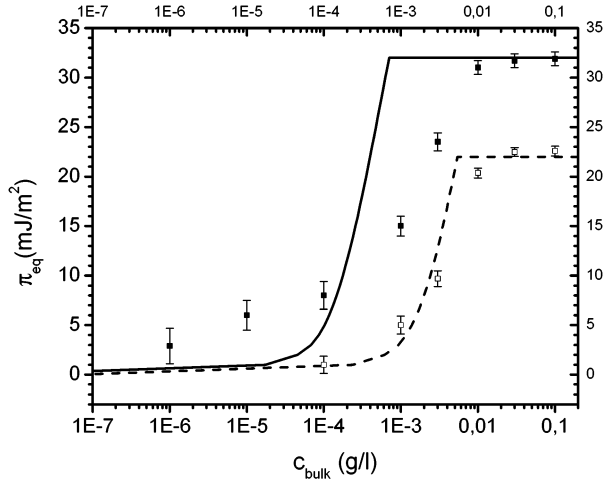

Figure 3. Experimental $\pi-c$ isotherms for β-casein at the air−water (open symbols) and the tetradecane−water (solid symbols) interface and theoretical curves for the air−water (dashed line) and the tetradecane−water (solid lines) interface.

periods of time in which the adsorption was recorded (~20 h), the extrapolated values arise also very close to the final interfacial tension value obtained in the experiments. The final data considered in the isotherm are determined from the mean value of both approximations. In Figure 3, the adsorption isotherms obtained in this manner for the air−water and the tetradecane−water interfaces are shown.

Similar adsorption isotherms have been obtained by Makievski et al. who focus their study on comparing the pendant drop film balance with conventional techniques.[18] Subsequently, the objective of this work is directed toward the effect of the nature of the interface on the interfacial structure adopted by the protein.

Important differences between the interfacial behavior of β-casein at air− and oil−water interfaces can be assumed by the experimental results shown in Figure 3. On one hand, the isotherm appears displaced to lower bulk concentration values just as the equilibrium interfacial pressure appears higher for the same bulk protein concentration at the tetradecane−water interface. On the other hand, the equilibrium interfacial pressure for the higher bulk concentrations appears much higher for the tetradecane interface, being 22 and 32 mJ/m^2 for the air and the oil interface, respectively.

In view of the experimental results, it can be elucidated that not only the forces that drive the protein onto the interface are modified by the oil interface but also the unfolding process undertaken by the adsorbing protein at the cited interface may differ from that at the air interface.

Although diverse explanations related to this question can be found in the literature, it still remains unclear.[2,9,19] To shed light on this matter, we put forward the following arguments as possible clarifications concerning the differences found in the protein adsorption process at both interfaces: On one hand, the oil appears as a better solvent than air for the more hydrophobic parts that hence can penetrate in the oil phase. In this sense, even at very high interfacial coverage, the unfolding process as well as the adsorption of new molecules could proceed, leading to the higher interfacial pressure found for the same bulk concentration at the oil interface. On the other hand, due to the marked hydrophobic character of the oil, there is a clear repulsion between molecules of oil and the water ones. Given this unfavorable interaction between the two solvents, the protein tends to place itself at the interface, trying to spread out as much as possible (this argument is also pointed out by Anderson et al.[2]). In conclusion, these two features exposed together may result in a further unfolded state achieved by the protein at this interface. In this line, Chipot et al. provide also evidence for this argument in terms of the study of the interfacial behavior of a peptide at the hexane−water interface by means of molecular dynamic computer simulations. In particular, it reveals a preferred orientation of the molecule parallel to the interface and a merely slightly less favorable orientation in which part of the molecule is immersed into the oil phase.[6] Hence, at low interfacial coverage, the molecular unfolding is favored, and the molecule spreads upon adsorption at the interface. As the bulk concentration is further increased, the large amounts of molecules result in a restriction to the rearrangement and conformational change processes at the interface.[27]

Figure 3 shows how the saturation concentration is slightly shifted to lower bulk protein concentrations at the oil interface. This shift to lower concentrations with increasing polarity of the neighboring phase is also found by Wüstneck et al. and attributed to the improved solvation of the different apolar and polar parts of the protein molecules in the oil phase that results in a higher interfacial activity of the protein.[27]

Although the experimental results, as reported by their $\pi-c$ isotherms, can offer an approximate idea about protein adsorption at liquid interfaces from a macroscopic point of view, they do not provide direct quantitative information on the interfacial configuration adopted by the protein at interfaces. To this end, the data shown in Figure 3 have been analyzed within the thermodynamic model of protein adsorption described in section II. To implement this theory, a fitting program has been developed by using an iterative technique. The starting point of the computer process resides in eq 4 rewritten as follows:

$$\Gamma_\Sigma = \frac{1}{\omega_\Sigma}\left[1 - \exp\left(a_{el}\Gamma_\Sigma^2\omega_\Sigma^2 - \frac{\pi\omega_\Sigma}{RT}\right)\right] \qquad (8)$$

Then, for a given value of π corresponding to a protein concentration below the critical value, an initial guess $\Gamma_\Sigma^{(1)}$ is supposed as a solution for the total adsorption. Therefore, taking into account the parameters n, ω_1, α, and a_{el} as input parameters in eqs 3 and 8, the right-hand side of eq 8 with $\Gamma_\Sigma^{(1)}$ provides a new guess $\Gamma_\Sigma^{(2)}$ for Γ_Σ. Subsequently, a new input for Γ_Σ can be formed from the following expression:

$$\Gamma_\Sigma^{(k+1)} = \lambda^{(k)}\Gamma_\Sigma^{(k)} + [1 - \lambda^{(k)}]\Gamma_\Sigma^{(k-1)} \qquad (9)$$

where $\lambda^{(k)}$ is a parameter of mix for the k-iteration, which must be chosen opportunely small for converging to the solution adequately. The iterative process is continued until the difference between two consecutives solutions for Γ_Σ is depreciative. This method has been successfully employed in the past for solving numerical systems based on integral equations.[28]

Once a value of Γ_Σ is obtained for the pressure considered, the protein concentration is calculated easily from eqs 2 and 5. For the case of protein concentrations above the critical value, taking into account $\omega_\Sigma = \omega_1\Gamma_\Sigma^*/\Gamma_c$ in eqs 6 and 7, a constant pressure value is derived for this range of concentrations.

According to these premises, Figure 3 shows the theoretical predictions obtained for the case of β-casein at the air−water (dashed line) and the tetradecane−water (solid lines) interfaces. The input parameters are taken as reported by the authors in ref 14, and they are shown in Table 1. As can be seen in this figure, the theory provides an excellent accordance with the experimental results at the air−water interface in agreement with

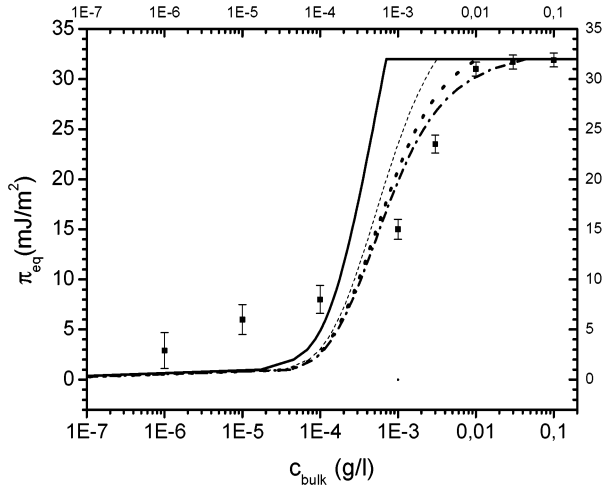

Figure 4. Effect of an increase in the fitting parameter ω_1 on the adsorption curve at the tetradecane−water interface: (—) $\omega_1 = 5$ nm^2/molecule; (− − −) $\omega_1 = 8$ nm^2/molecule; (···) $\omega_1 = 9$ nm^2/molecule, (− · −) $\omega_1 = 9.5$ nm^2/molecule.

TABLE 1: Fitting and Input Parameters Used in Figure 3

	air−water	tetradecane−water
b_1 (L/mol)	6.9×10^{-6}	9.4×10^{-7}
a_{el}	70 ± 20	70 ± 20
α	0.00	0.00
ω_{max} (nm^2/molecule)	100 ± 20	100 ± 20
ω_{min} (nm^2/molecule)	5 ± 2	5 ± 2

previous studies.[12,13,16] However, at the tetradecane−water interface, the agreement with the experimental results appears not as satisfactory with this set of parameters. This feature is not in fact surprising taking into consideration the experimental results presented above, which account for a slightly further unfolded structure attained by β-casein at the tetradecane−water interface. Therefore, it seems essential to introduce this feature into the theoretical treatment by increasing the minimum interfacial molecular area occupied by the protein at the interface. Figure 4 shows the effect on the theoretical isotherm of an increase in ω_{min}. It can be seen how the fitting clearly improves as the minimum interfacial molecular area of the protein increases, thus accounting for the experimental findings.

Bearing in mind the proven validity of the theoretical model used in this study at the air−water interface with several proteins,[1,16] the theoretical predictions in Figure 4 provide reliable quantitative information of the unfolding process undertaken by the protein at the oil interface. Accordingly, the application of this adsorption model not only corroborates but quantifies the experimental findings. With respect to the lower part of the isotherm, the behavior of which is not as satisfactorily reproduced, further fittings as well as the application of a modification of the model are under study.

According to the plan exposed in the Introduction, the study is completed by applying the model to some results by Graham and Phillips.[17] In particular, Figure 5 shows a comparison between our data of adsorption of β-casein at the tetradecane−water interface with those performed by these authors with the same protein at the toluene−water interface and using a Langmuir trough.

As can be seen, both experimental isotherms appear located in the same bulk concentration region but differ in the shape and also in the maximum π attained. The latter question is adequately addressed by Makievski et al. in terms of the geometrical differences arising from both experimental techniques.[18] Concerning the differences in the shape of the isotherm,

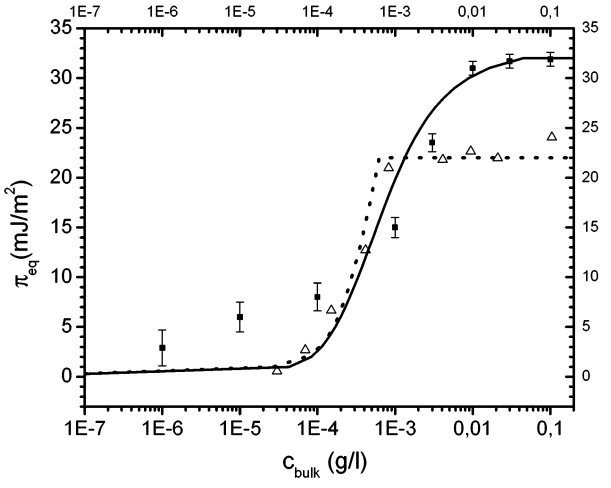

Figure 5. Experimental $\pi-c$ isotherms for β-casein at the tetradecane−water (■) and the toluene−water (△) interface[15] and theoretical curves for the tetradecane−water (—) and the toluene−water (···) interface.

TABLE 2: Fitting and Input Parameters Used in Figure 5

	toluene−water	tetradecane−water
b_1 (L/mol)	6×10^{-7}	9.4×10^{-7}
a_{el}	70 ± 20	70 ± 20
α	0.00	0.00
ω_{max} (nm^2/molecule)	100 ± 20	100 ± 20
ω_{min} (nm^2/molecule)	5 ± 2	9.5 ± 2

discrepancies may derive from the different oil phase used. In this sense, the application of the adsorption model provides information on the structural configuration adopted by the protein at both interfaces. Concretely, Figure 5 shows the theoretical predictions corresponding to the fitting parameters displayed in Table 2 where the protein unfolding extent is quantified by ω_1. Accordingly, the values $\omega_1 = 5$ nm^2/molecule and $\omega_1 = 9.5$ nm^2/molecule for β-casein at the toluene− and the tetradecane−water interfaces, respectively, account for less unfolding undertaken by the protein at the former interface. This result agrees with Graham and Phillips findings who report that the flexible protein β-casein adopts a further expanded state at the air−water interface with respect to the toluene−water interface.[7,17] Since the unravelling of the protein at the interface proceeds to decrease the surface energy,[11] a possible explanation of the differences between the interfacial structure of the protein at both interfaces might be related to the differences found in the interfacial tension values. The interfacial tension of the toluene−water interface (35 mJ/m^2) is lower than that of the tetradecane−water interface (53 mJ/m^2).[29] In view of this, the less interfacial tension found at the toluene−water interface might be responsible for the less unfolded state of β-casein described by Graham and Phillips at the cited interface, which is also corroborated by the fitting procedure.

V. Conclusion

A procedure of analysis of protein adsorption isotherms at liquid interfaces is proposed in terms of the $\pi-c$ curves and a thermodynamic model. Comparison of the experimental $\pi-c$ data for β-casein adsorption at the air− and the tetradecane−water interfaces suggest that the nature of the interface significantly affects the adsorption process. At this oil interface, a further penetration of the adsorbing protein into the oil phase can be assumed at higher bulk concentrations. Concretely, in view of the experimental data at the air interface, β-casein seems to adopt a further unfolded configuration at the tetradecane

interface. However, analysis of the experimental isotherms does not provide quantitative information on this feature. For this reason, a thermodynamic model recently developed by Makievski et al. for protein adsorption is applied directly to the $\pi-c$ curves. The application of this model with the subsequent change in the interfacial area parameters at both interfaces corroborates the experimental findings. Furthermore, by means of this procedure the protein unfolding extent is quantified at both interfaces. In addition, to evaluate the effect of the nature of the interface on the protein unfolding process, the cited theory is applied to Graham and Phillips results for β-casein at the toluene−water interface.[15] As a result, the fitting parameters confirm the further unfolded state achieved by this protein at the tetradecane−water interface observed experimentally.

Another remarkable aspect of this work, lies in the fact that, to our knowledge, it is the first application of a theoretical model to protein $\pi-c$ adsorption curves onto an oil interface. In consequence, the treatment given in this work to the experimental data offers many possibilities in the clarification of the adsorption of proteins onto liquid interfaces. Considering relatively simple experiments, as the acquisition of $\pi-c$ isotherms, the application of this thermodynamic model provides quantitative information of the structure adopted by the protein at the interface, as well as on the interaction with the nonpolar phase.

Acknowledgment. Financial support from "Ministerio de Ciencia y Tecnología, plan nacional de Investigación científica. Desarrollo e Innovación Tecnológica (I+D+I)", proyects MAT2001-2834-C02-01, AGL2001-3483-C02-02, and Plan Propio de la Universidad de Granada are gratefully acknowledged.

References and Notes

(1) Möbius, D., Miller, R., Eds. *Proteins at liquid interfaces*, 1st ed.; Elsevier: Amsterdam, 1998.

(2) Anderson, R. E.; Pande, V. S.; Radke, C. J. *J. Chem. Phys.* **2000**, *112*, 9167.

(3) Douillard, R.; Daoud, M.; Aguié-Béghin, V. *Curr. Opin. Colloid Interface Sci*. **2003**, *8*, 380.

(4) Benjamins, J.; Lucassen-Reynders, E. H. In *Proteins at liquid interfaces*, 1st ed.; Mobius D., Miller R., Eds.; Elsevier: Amsterdam, 1998; Chapter 9, p 303.

(5) Dickinson E., Van Vliet, T., Eds. *Food Colloids, Biopolymers and Materials*; Royal Society of Chemistry: Cambridge, U.K., 2003.

(6) Chipot, C.; Pohorille, A. *J. Am. Chem. Soc.* **1998**, *120*, 11912.

(7) Graham, D. E.; Phillips, M. C. *J. Colloid Interface Sci.* **1979**, *70*, 427.

(8) Beverung, C. J.; Radke, C. J.; Blanch, H. W. *Biophys. Chem.* **1999**, *81*, 59.

(9) Segumpta, T.; Razumovsky, L.; Damodaran, S. *Langmuir* **1999**, *15*, 6991.

(10) Murray, B. S.; Nelson, P. V. *Langmuir* **1996**, *12*, 5973.

(11) Cai, J.; Prausnitz, J. M. *J. Chem. Phys.* **2002**, *117*, 3935.

(12) Fainerman, V. B.; Miller, R.; Wüstneck, R. *J. Colloid Interface Sci.* **1996**, *183*, 25.

(13) Makievski, A. V.; Fainerman, V. B.; Bree, M.; Wüsteck, R.; Krägel, J.; Miller, R. *J. Phys. Chem. B* **1998**, *102*, 417.

(14) Fainerman, V. B.; Miller, R. *Langmuir* **1999**, *15*, 1812.

(15) Joos, P.; Serrien, G. J. *J. Colloid Interface Sci.* **1991**, *145*, 291.

(16) Miller, R.; Fainerman, V. B.; Makievski, A. V.; Krägel, J.; Grigoriev, D. O.; Kazakov, V. N.; Sinyachenko, O. V. *Adv. Colloid Interface Sci.* **2000**, *86*, 39.

(17) Graham, D. E.; Phillips, M. C. *J. Colloid Interface Sci.* **1979**, *70*, 415.

(18) Makievski, A. V.; Miller, R.; Fainerman, V. B.; Krägel, J.; Wüsteck, R. In *Food Emulsions and Foams*; Dickinson, E., Rodriguez-Patino, J. M., Eds; Royal Society of Chemistry: Cambridge, U.K., 1999; Chapter 22, p 269.

(19) Russev, S. C.; Arguirov, T. V.; Gurkov, T. D. *Colloids Surf., B* **2000**, *19*, 89.

(20) Lucassen-Reynders, E. H. *J. Phys. Chem.* **1966**, *70*, 1771.

(21) Lucassen-Reynders, E. H. *J. Colloid Interface Sci.* **1972**, *41*, 156.

(22) Cabrerizo-Vílchez, M. A.; Wege, H. A.; Holgado-Terriza, J. A. *Rev. Sci. Instrum.* **1999**, *70*, 2438.

(23) Graham, D. E.; Phillips, M. C. *J. Colloid Interface Sci.* **1979**, *70*, 403.

(24) Tripp, B.; Magda, J. J.; Andrade, J. D. *J. Colloid Interface Sci.* **1995**, *173*, 16.

(25) Fainerman, V.; Makievski, A. V.; Miller, R. *Colloids Surf., A* **1994**, *87*, 61.

(26) Wüstneck, R.; Krägel, J.; Miller, R.; Wilde, P. J.; Sarker, D. K.; Clark, D. C. *Food Hydrocolloids* **1996**, *10*, 395.

(27) Wüstneck, R.; Moser, B.; Muschiolik, G. *Colloids Surf., B* **1999**, *15*, 263.

(28) Martín-Molina, A.; Quesada-Pérez, M.; Galisteo-González, F.; Hidalgo-Álvarez, R. *J. Phys. Chem. B* **2002**, *106*, 6881.

(29) Weast, R. C.; Selby, S. M.; Long, J. W.; Sunshine, I. *Handbook of Chemistry and Physics*, 55th ed.; CRC Press Inc.: Cleveland, OH, 1974.