Thermochimica Acta, 162 (1990) 1–16
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PROTEINS SOLUBILIZED IN ORGANIC SOLVENTS VIA REVERSE MICELLES: THERMODYNAMIC STUDIES *

P.L. LUISI and G. HÄRING

Institut für Polymere, Eidgenössische Technische Hochschule Zürich, ETH-Zentrum, 8092 Zürich (Switzerland)

M. MAESTRO

Dipartimento di Chimica, Università di Bari, Via Amendola 173, 70126 Bari (Italy)

G. RIALDI

Centro di Studi Chimico-fisici di Macromolecole Sintetiche e Naturali, C.N.R., C.so Europa 30, 16132 Genova (Italy)

(Received in final form 28 December 1989)

ABSTRACT

The field of reverse micelles and, in particular, of proteins solubilized in reverse micelles is briefly reviewed, with emphasis on the system AOT in isooctane (where AOT is bis(2-ethylhexyl)sodium sulfosuccinate). It is then shown how experimental and theoretical thermodynamical approaches can be applied to micelle systems. First, the question of the amount of water bound to AOT is tackled by calorimetry. Depending on whether w_0 ($w_0 = [H_2O]/[AOT]$) is below or above 10, an endothermic or an exothermic transition is observed. The data show that 6 water molecules are firmly bound to each polar AOT head group, resulting in unfreezable water. Microcalorimetry is then applied to study the thermodynamic stability of proteins present in the pool water of some reverse micelles (lysozyme, cytochrome-c, ribonuclease). It is shown that the presence of the protein decreases the apparent C_p at any given temperature, and that the unfolding temperature of the protein depends on w_0 and decreases with increasing water content. The thermodynamic stability of proteins in reverse micelles is lower than that in aqueous solution, and the corresponding enthalpic and entropic contributions of the free energy of the transition are interpreted in terms of the micellar structure. Finally, as a last example of the application of theoretical thermodynamics to the protein-containing reverse micelles, the question of the driving forces responsible for solubilization of their proteins is discussed. A thermodynamic treatment underlying this process is put forward. It is shown that the free energy change can be explicitly written, and that its minimization permits calculation of important structural micellar parameters such as the radius of the filled and unfilled micelles.

---

* Presented at the 10th AICAT, Pisa, Italy, 11–14 December 1988.

0040-6031/90/$03.50 © 1990 – Elsevier Science Publishers B.V.

INTRODUCTION

Certain surfactants dissolved in apolar solvents form spherical aggregates, in which the polar head groups of the surfactant molecules are directed towards the inside, defining a polar core, and the lipophilic tails are directed towards the bulk solvent. Water can be solubilized in the polar core, thus forming a water pool in which hydrophilic molecules can then be solubilized. The system isooctane–AOT–water (where AOT is bis(2-ethylhexyl)sodium sulfosuccinate), has been the subject of particular attention. The size of these aggregates, as well as several of their physical properties, depend primarily on the molar ratio of water to surfactant, $w_0 = [H_2O]/[AOT]$. In the w_0 range 5–50, the radius of the water pool is 10–80 Å [1,2]. At low values of w_0, the term "reverse micelle" is used, while at high w_0 values (e.g. greater than 15) the term "water in oil" (w/o) microemulsion is more correct. In this and many other papers, however, the first term has been adopted as a useful generalization.

The physical properties, including the thermodynamics and the kinetics of micelle formation, have been the subject of recent research [2–6]. In particular, physico-chemical studies have revealed that reverse micelles are highly dynamic systems, able to coalesce either on collision with each other, or with exchange materials contained in the water pools [7,8]. According to some researchers, these structures are also biologically important, as they can be formed in vivo in lipid structures [9,10].

The properties of water in the water pool have been studied with particular attention, as it appears that some of its physical properties are significantly different from those of bulk water [11,12]. This, of course, is very important for reactivity studies (including enzymatic reactions) in reverse micelles. For example, in reverse micelles at low w_0 values water has a freezing temperature well below zero, which permits cryoenzymology to be performed [13].

About 10 years ago, there was a sharp increase in interest owing to the finding that hydrophilic enzymes can be solubilized in reverse micellar systems and still maintain their activity [14,15]. Since then, work on the solubilization of proteins and enzymes in reverse micellar solution has increased steadily, and many reviews on the subject have already been published (for example, see refs. 16–18).

Current work is primarily oriented in two directions: basic studies, intended to clarify the conformation and the mechanisms of action of enzymes in this new environment; and biotechnology studies, indended to exploit these macromolecular systems for industrial applications, for example for the catalytic transformation of water-insoluble substrates [19,20] or for protein extraction and purification [21,22]. Nucleic acids [23] and entire cells [24] can also be solubilized in organic solvents via reverse micellar solutions, and it has been shown that microemulsions can be transformed into gels having a very high viscosity (microemulsion gels) [25,26].

This paper will examine some work dealing more directly with thermodynamic problems. In particular, three topics will be considered: calorimetric studies for the determination of the amount of water bound to the AOT micellar wall; microcalorimetric studies on protein-containing reverse micelles, aimed at determining the stability of such macromolecular host-guest systems; and a thermodynamic approach aimed at illustrating the driving forces responsible for protein solubilization in reverse micelles.

THE QUESTION OF WATER BOUND TO AOT IN REVERSE MICELLES

We have already mentioned that water in the restricted environment of the reverse micelle displays some anomalies with respect to bulk water: examples include its activity [27], its local dielectric constant [28] and the NMR chemical shift of its protons [11], as well as its IR resonance stretching [12].

This is a result of the fact that some of the water in the AOT micelle is bound to the sulfonate group. A few studies on the interaction between water and AOT have been carried out, but there is still uncertainty about the precise stoichiometry of the system.

We have recently applied microcalorimetry to this problem [29]. The procedure is as follows: a reversed micellar dispersion (50 μl) containing 20 mM, 50 mM or 100 mM AOT is injected into a stainless steel DSC pan which is sealed immediately. In the reference pan a corresponding solution of AOT in isooctane without H_2O is used. The samples are cooled to about $-30\,^{\circ}$C and then heated. The peak in the heat capacity versus temperature curve is then integrated to obtain the transition enthalpy ΔH°.

Reversed micelles with $w_0 < 10$ showed no exothermic event when cooled. The heating step gave an endothermic transition at $0\,^{\circ}$C. This was assigned to the melting of ice. When the same procedure was applied to AOT reverse micelles containing more water ($w_0 > 10$), an exothermic transition was observed (Fig. 1). Large hysteresis effects were observed. The temperature of ice formation decreased with decreasing water content (w_0), and the enthalpy ΔH° associated with this event showed the same trend. Extrapolating a plot of the enthalpy ΔH° of the ice melting peak as a function of w_0 to $\Delta H^{\circ} \rightarrow 0$ gave a value of $w_0 = 6.1 \pm 0.2$ as the intercept on the w_0 axis (see Fig. 2). This value may be identified as the amount of unfreezable water. The value of 76 ± 3 cal g^{-1} derived in this way is in reasonably good agreement with the theoretical value of 79.7 cal g^{-1}. From this result we can conclude that all the water, except for about six water molecules per AOT molecule, freezes and forms ice upon cooling. The unfreezable water is so strongly bound to AOT that the formation of ordinary ice is prevented.

The stoichiometry of the bound water obtained by this thermodynamic treatment is in good agreement with data in the literature based on other methods [30,31].

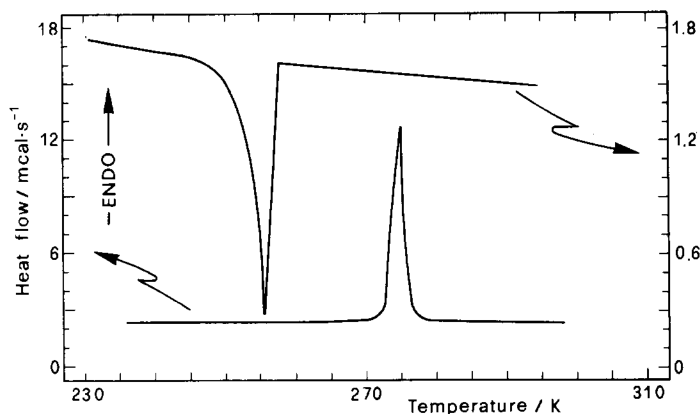

Fig. 1. Heating and cooling differential scanning calorimetry (DSC) curves for AOT reverse micelles in isooctane, recorded at 2.5 °C min^{-1}, [AOT] = 150 mM, $w_0 = [H_2O]/[AOT] = 50$.

In a paper describing the calorimetric method, spectroscopic methods were also applied to determine the stoichiometry of the water bound to AOT in reverse micelles, in particular deuterium NMR, ESR and proton NMR methods [29]. All these data are in substantial agreement, indicating an

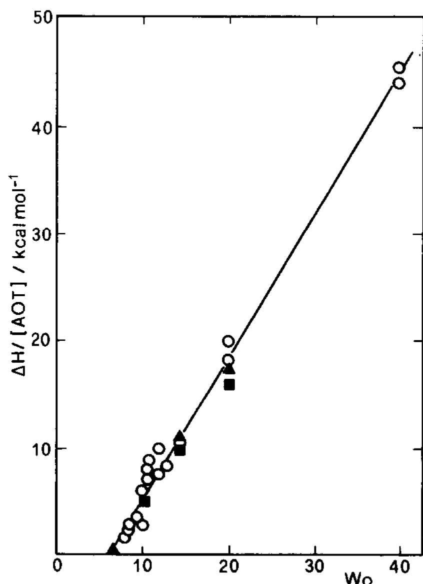

Fig. 2. Normalized enthalpies $\Delta H/[AOT]$ as a function of the water content ($w_0 = [H_2O]/[AOT]$) of AOT reverse micelles in isooctane. The enthalpy ΔH of the melting of ice was determined by DSC after cooling the AOT reverse micelles to about −30 °C. Reverse micelles of different AOT concentrations were used: ■, 20 mM AOT; ▲, 50 mM AOT; and ○, 100 mM AOT. Their water content w_0 was from 6 to 40.

amount of bound water in the range of 5–15 molecules of water bound per AOT head-group; however, the agreement is not perfect, indicating that each experimental method weighs the amount of bound water differently.

THERMODYNAMIC STUDY ON GLOBULAR PROTEIN STABILITY IN REVERSE MICELLES

The previous studies were carried out on "unfilled" AOT reverse micelles, i.e. those containing no protein. It is perhaps relevant to recall at this point the main ideas current in the field concerning the structure of protein-containing reverse micelles.

On the basis of spectroscopic evidence (UV, circular dichroism and fluorescence), globular proteins are assumed to be located inside the water pool. This situation is best described by the so-called "water-shell" model [32], according to which the protein molecule is surrounded by one or more layers of water molecules protecting the protein from the denaturating effects of isooctane and AOT molecules. Enzymes such as α-chymotrypsin and ribonuclease are in fact still active in the AOT micelles [14,17]. At low amounts of water, the volume of the micellar water pool is comparable to the hydration shell of small globular proteins, and under such conditions some novel properties of the guest enzymes may be found. For example, at low w_0 the specific activity of α-chymotrypsin [15,33,34] and peroxidase [35] increases several times with respect to the activity in aqueous solution. Significant increases in the temperature stability are also observed.

Clearly, it would be very interesting to gather information on the amount of water bound to proteins inside the micelle. One may expect competition between the AOT wall and the protein for the water in the water pool, and a comparison with the case of unfilled micelles could given valuable information on the stoichiometry of the water bound to proteins. This kind of experiment is, however, rather difficult to conduct and up until now only a few preliminary studies on "filled" micelles (i.e., those containing protein) have been carried out to assess the relative stability of the protein. The thermal unfolding of proteins can be measured by microcalorimetry. The question then becomes: is there a difference in thermal stability between the protein in bulk water and that in the reverse micelle?

The unfolding transition of three small globular proteins (ribonuclease, cytochrome-c and lysosyme) in the AOT–isooctane micellar system has recently been studied by differential scanning calorimetry [36]. The thermodynamic parameters associated with the transition were studied as a function of the critical parameters and system composition of the micelle.

The experiment was performed as follows: calorimetric cells (stainless steel cells) were filled by weight, cooled to the desired temperature, and then warmed at a rate of 28 °C h^{-1}.

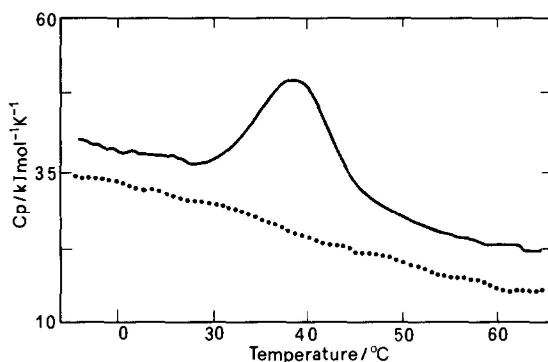

Fig. 3. Scanning calorimetric recording of the AOT–isooctane micellar system at $w_0 = 11.1$. The lower curve was recorded for a 100 mM AOT–isooctane solution without protein. The same micellar solution, this time containing 4 mg ml^{-1} of ribonuclease in 10 mM diglycine, pH 3.36, produced the upper curve. The reference cell was filled with pure isooctane. The scan rate was 28 °C h^{-1}. W_0 is the molar ratio of water and AOT ($w_0 = (H_2O]/[AOT])$.

The apparent C_p of the micelles containing water displayed a negative temperature coefficient. On the other hand, at a given temperature the apparent C_p of the micellar solution increased as a function of the water content. Addition of the protein to the micellar solution slightly increased the apparent C_p at any given temperature (Fig. 3). Furthermore, a sharp transition was observed between 10 °C and 80 °C. The intense heat absorption effect probably results from the presence of the protein, and may be assigned to a cooperative transition of the macromolecule.

Figure 4 shows the transitions observed in aqueous solution and in AOT reverse micelles at different w_0 values, as obtained from the best fit of the experimental curves after baseline subtraction. It appears that the thermal

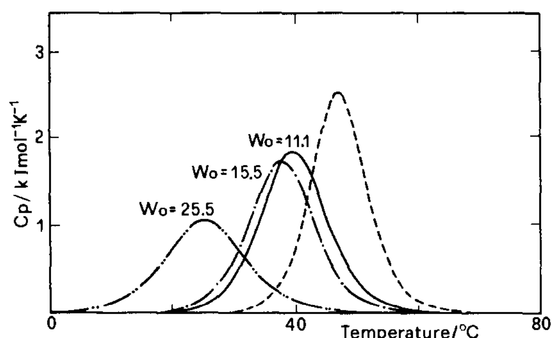

Fig. 4. Temperature dependence of the apparent molar heat capacity of ribonuclease in water and as a function of w_0. Aqueous solutions: 10 mM diglycine, pH 3.36 (— — —); and 100 mM AOT–isooctane with the same buffer at $w_0 = 11.1$ (———), 15.5 (·–·–·) and 25.5 (–··–).

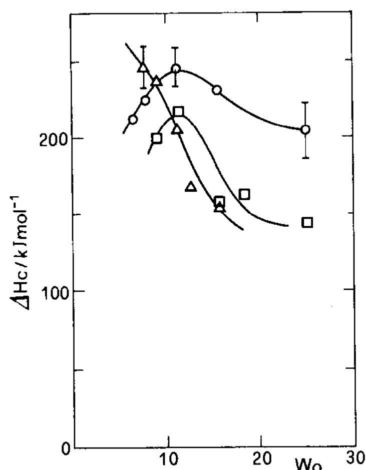

Fig. 5. Dependence on w_0 of the apparent calorimetric ΔH of the transition of ribonuclease, cytochrome-c and the lysozyme–NAG_3 complex. 100 mM AOT–isooctane–diglycine 10 mM, pH 3.36, containing ribonuclease (○), cytochrome-c (□), and the lysozyme–NAG_3 complex (△) ($[NAG_3] = 0.03$ mM). The error bars give the experimental uncertainty from at least three measurements.

stability of the proteins hosted in reverse micelles depends on the water content, and decreases when w_0 increases (i.e., the bigger the water pool).

The calorimetric enthalpic contribution of the transition of the protein micellar system is dependent on w_0 (Fig. 5). For ribonuclease and the lysozyme–NAG complex (NAG is the inhibitor tri-*N*-acetylglucosamine), it passes through a maximum with its highest T_m and ΔH_c at w_0 values in the range 9–11.

Thus, as the size and water content of the micelles increase, the stability of the protein as defined by T_m decreases. At first sight this is a surprising result, as we are dealing with water-soluble enzymes which usually display their optimal activity in aqueous solutions. However, one should recall that the activity of most globular enzymes in reverse micelles has a maximum in the w_0 range 8–12, and decreases when there is a further increase in the water content [14,18]. Thus, it seems possible to correlate activity with thermodynamic stability: when w_0 increases above a critical value, the stability of the proteins in reverse micelles tends to decrease, which brings about a decrease in activity.

It is then clear that the stability of proteins in reverse micelles, as well as their activity, critically depend on the nature of the water in the water pool. Water behaves in a rather peculiar way when it is present mostly in a bound form (as we saw in the previous section, in this w_0 range (8–12) water is mostly hydration water), and this also affects the properties of the guest macromolecules. It should also be noted that the w_0 region of greatest stability corresponds to that of the highest protein solubility in the reverse

TABLE 1

Thermodynamic parameters for the transition of ribonuclease and cytochrome-c in aqueous and AOT–isooctane micellar solution (at 25 °C and pH 3.3) from ref. 36

	Type of solution	ΔH (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})
Ribonuclease	water	177.8	160	17.8
	W_0 25.5	198	196	2.0
	W_0 11.1	208	198	10
Cytochrome-c	water	39	21.4	17.6
	W_0 15.7	127	124	3
	W_0 9.2	143.3	134	9.3

micellar system [22], i.e. the solubility drastically decreases at higher w_0 values.

The thermodynamic treatment illustrated thus far also allows an initial estimate to be made of the energy parameters associated with the unfolding of the proteins (Table 1).

The calculated free energy change at 25 °C and $w_0 = 11.1$ for ribonuclease is lower in micelles than in aqueous solution, but is still positive. The entropy and enthalpy changes for the cooperative process in reverse micelles are larger than those in water. In other words, the free energy difference between the folded and thermally unfolded state is smaller in micelles than in water. From the data of Table 1, it is apparent that this is a result of an unfavorable increase in the $-T\Delta S$ term not sufficiently compensated for by the enthalpic term.

Similar increases in the ΔH and ΔS of unfolding have also been observed for other proteins in non-aqueous media [37–39] and have been ascribed to solvation changes. Can the larger entropic contribution in our case be ascribed to water solvation?

This hypothesis can be discussed on the basis of two considerations, originally presented in ref. 36. One explanation might be seen in the different hydrophobic effects in micelles as opposed to water. In micelles at $w_0 = 11$, the water in the water pool is mostly bound, and unfolding is attended by smaller hydrophobic interactions, since there is a lower ordering effect of the water molecules. This difference may be supposed to be reflected in more positive contributions to both the enthalpic and entropic terms in micelles.

The second possible source of positive entropic contribution is associated with the changes in the hydration of the protein and/or micellar wall. Uptake of a protein causes redistribution of the water molecules of the water pool, and it is essentially an entropy-driven process [40]. Since unfolding appears to be associated with a decrease in the bound water [41], there would be a smaller entropic gain in micelles at $w_0 = 11$ than in bulk water.

This interpretation would also explain the difference between the two proteins in Table 1, since cytochrome-c appears to be preferentially located in the micellar wall rather than in the middle of the water pool [42].

Comparison of the data at w_0 values of 11.1 and 25.5 (Table 1) is also very informative. Unfolding becomes more favorable with a higher water content, as we have already seen. It would in fact be interesting to evaluate the thermodynamic parameters in the smaller w_0 region—it is possible that at a w_0 value smaller than 11.1 the protein in micelles becomes as stable as that in water. This is conceivable in view of the fact that enzymes in reverse micelles appear to exhibit their maximal activity and conformational stability primarily at very low levels of water content. However, this experiment cannot be accomplished by calorimetry due to insufficient solubility; indeed, this is one of the limits of the technique.

The transition temperatures under various micellar conditions are investigated in ref. 36.

A THEORETICAL TREATMENT

Many questions in the field can probably only be answered by a thermodynamic theory. The first question relates to the driving forces responsible for the uptake of proteins into reverse micelles. The efficiency of micellar systems in extracting proteins (even hydrophilic proteins) from aqueous systems, or from the solid state, is remarkable [22,43] and indeed incomprehensible at first sight.

The question of the driving forces is connected, of course, to all the structural aspects already discussed above—the state of the water, the water solvation, and the conformational changes experienced by the protein inside the micelle.

To treat this problem thermodynamically is not an easy matter, one reason being the many system variables involved (the surfactant, the water associated with the micelle, protein, salt and ion concentrations, etc.). Ideally, one would like to have a thermodynamic scheme which gives the final micellar parameters from these initial conditions and, in particular, the enthalpic and entropic energy changes associated with the uptake of the protein, and the structural and stoichiometry parameters defining the final state (e.g. the dimension of the protein-containing micelle, the stoichiometry of the bound water, the distribution of the filled and unfilled micelles, etc.).

An approximate thermodynamic model partially answering some of these questions has been proposed by Maestro et al. [40,44].

The main idea is that electrostatic interactions play the most important role in the forward transfer of a protein from an aqueous phase to a supernatant micellar solution. The interactions between the protein (assumed to be positively charged) and the negatively charged shell of the AOT constitute the most important contribution. The driving forces resulting

from such an effect will be primarily entropic; when the protein is incorporated into the micelle, pairs of counterions will be expelled from the micellar wall and from the protein surface into the bulk water phase, thus giving rise to a positive entropy change.

From a mathematical point of view, Maestro et al. make use of the cell method: thus, the whole bulk space is divided into spherical regions (cells), each with one protein molecule in the center.

The problem of determining the amount of protein being transferred from one phase to the other is then reduced to a determination of the conditions for the equilibrium state. This implies a minimization of the free energy change, calculated as the difference between a given intermediate state and a precisely defined reference state. A physical model is needed for these energy calculations. The basic idea is to consider the reverse micelle as a capacitor, with one negatively charged wall (the AOT sulfonate ions) and a layer of overfacing counterions. Uptake of the protein into a reverse micelle thus becomes a matter of building up a system of capacitors, one inside the other. The inner capacitor (see Fig. 6) is the protein, which has a positively charged surface faced by a layer of negatively charged counterions.

This model has been applied to two experimental situations: the so-called phase transfer [44], in which the protein is transferred from an aqueous solution to a supernatant micellar hydrocarbon solution; and the injection technique [40], in which the aqueous protein solution is injected into the organic micellar solution using a microsyringe. The thermodynamic scheme is the same for these two cases, but our equation refers to the second one, which is the most common experimental method for the preparation of protein-containing reverse micelles.

The total free energy change is the sum of the following four contributions.

(1) An electrostatic interaction energy: upon uptake of the protein, there will be a redistribution of materials and of the ions, and therefore a different electrostatic interaction involving the polyions (protein and micellar wall). This energy term can be expressed as follows:

$$\Delta\mathcal{G}^{\circ}_{\mathrm{Ee}} = \left(|z_1| Q X_{\mathrm{a}}/\epsilon_{\mathrm{p}}\right)\left\{z^2[z/(z-l) - y/(y-l)] + b[y/(y-l) - g/(g-l)]\right\} \quad (1)$$

where $\Delta\mathcal{G}^{\circ}_{\mathrm{Ee}} = \Delta G^{\circ}_{\mathrm{Ee}}/NkTn_{\mathrm{p}}V$ is the reduced free energy variation per mole of dissolved protein and where we have introduced the following notations:

$$Q = q^2 l X_{\mathrm{a}} |z_1| / (8\pi\epsilon_0 kTr_{\mathrm{p}}) \quad (2)$$

$$X_{\mathrm{a}} = 4\pi r_{\mathrm{p}}^2 |z_{\mathrm{a}}| / (S_{\mathrm{a}} |z_1|) \quad (3)$$

where $|z_1|$ is the absolute value of the surfactant counterion charge, $|z_{\mathrm{a}}|$ is the absolute value of the surfactant polar head charge, l is the dimensionless

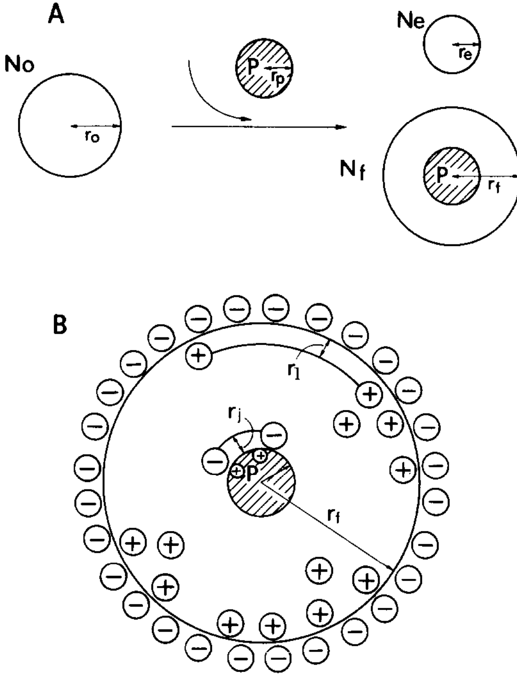

Fig. 6. A, Schematic representation of the protein uptake by reverse micelles, with indication of the main geometrical parameters utilized in the computational scheme. Following the uptake of N_f protein molecules with radius r_p, the system re-equilibrates and the monodispersed population of N_0 empty micelles with radius r_0 is irreversibly transformed into a bimodal population of N_e unfilled and N_f filled micelles with radii r_e and r_f, respectively. B, The protein-containing reverse micelles seen as a system of two interacting concentric microcapacitors. This has been utilized in the scheme primarily to calculate the electrostatic energy contributions to the free energy change. Note the two geometrical parameters r_l and r_j; these represent the thickness of the microcapacitors, which are taken as being equal to the radii of the AOT and protein counterions, respectively.

ratio r_l/r_p and q is the elementary charge. (A list of the symbols used is given at the end of the paper.)

(2) The corresponding electrostatic entropy term: this can be understood by recognizing that polyions attract an increased concentration of counterions near their surfaces, thus giving rise to a non-homogeneous distribution of mobile charges and, consequently, to a decrease in entropy (with respect to an ideal solution in which polyions are not present). The corresponding contribution can be expressed as follows:

$$\begin{aligned}\Delta\mathcal{G}^{o}_{Se} = {} & (b - z^2) X_a \ln\{y^2(y - X_a l^3)/[y^3 - (y - 2l)^3]\} \\ & + X_a z^2 \ln\{E/[z^3 - (z - 2l)^3]\} + X_p \ln\{E/[(2j + 1)^3 - l]\} \\ & - bX_a \ln\{g^2(g - X_a l^3)/[g^3 - (g - 2l)^3]\} \qquad (4)\end{aligned}$$

where $\Delta\mathcal{G}^{o}_{Se} = \Delta G^{o}_{Se}/NkTn_pV$ and $X_p = 4\pi r_p^2 |z_p| / S_p |z_j|$, $S_p = q/\sigma_p$ (Å^2).

The third and fourth contributions to the free energy difference are both mixing entropy changes. The first is a dilution entropy change, this is an ideal solution entropy change deriving from the possible exchange of water and mobile ions between the reverse micelles and the cells of the underlying water pools, the exchange being activated by the protein uptake. This term is distinct from the electrostatic entropy term, as it can also be present for uncharged mobile components. The second term is a mixing free energy change in which the reverse micelles are seen as particles in the oil phase.

If the relationships which are valid for simple ideal solutions are used for both terms, one can write:

$$\begin{aligned}\Delta\mathcal{G}^{\circ}_{id} = {} & X_a z^2\{\ln[(X_a z^2)/D] - 1\} + X_p[\ln(X_p/D) - 1] \\ & + bX_a\{\ln[1 + d(g - X_a l^3)] + 1\} \\ & - X_a(b - z^2)\{\ln[1 + d(y - X_a l^3)] + 1\} \\ & + (1 + X_p)\{\ln[1 + (G/X_p)(w - X_p j^3 - 1) + 1/X_p] + 1\} \\ & + \ln(1/D) - 1 - X_p \ln\{w/[(2j + 1)^3 - 1]\}\end{aligned} \tag{5}$$

$$\begin{aligned}\Delta\mathcal{G}^{\circ}_{mix} = {} & \ln\{y^2/[b - z^2 + y^2(L + 1)]\} + (b - z^2) \\ & /y^2(\ln\{(b - z^2)/[(b - z^2) + y^2(L + 1)]\} - 1) \\ & - b/g^2\{\ln[b/(b + g^2 L)] - 1\} - 1\end{aligned} \tag{6}$$

In these equations, we have introduced six new auxillary parameters:

$$G = 4\pi r_p^3/3v_w \tag{7}$$

$$d = G/X_a \tag{8}$$

$$L = n_0/n_p \tag{9}$$

$$D = X_a z^2 + X_p + GE + 1 \tag{10}$$

$$E = z^3 - X_a l^3 z^2 - X_p j^3 - 1 \tag{11}$$

$$w = n_a/Gn_p \tag{12}$$

where n_0 is the oil concentration (mol ml^{-1}), the meanings of $\Delta\mathcal{G}^{\circ}_{id}$ and $\Delta\mathcal{G}^{\circ}_{mix}$ are obvious, and $j = r_j/r_p$.

One can now minimize the total free energy change resulting from the sum of the four terms defined above. We refer the reader to the original paper for a complete description of the method.

CONCLUDING REMARKS

Thermodynamic studies can be successfully applied only when the questions can be clearly formulated and focused. In this sense, the fact that

experimental and theoretical thermodynamic treatments are applied to reverse micellar systems can be taken as a good indication of the maturity attained in this field.

Of course the answers which have been produced so far are only partial. This reflects both the present situation and the objective difficulties presented by the systems under investigation. This is particularly true of protein-containing reverse micelles; the existence of a bimodal distribution of the micelles (filled and unfilled) and their low degree of occupancy (i.e., the low relative concentration of filled micelles, especially at low w_0 values) makes it very difficult to obtain a complete set of calorimetric data and hence a complete picture of the situation. Nevertheless, the work of Battistel et al. [36] has undoubtedly shed new light on the subject and should be extended, particularly using proteins that are more soluble in reverse micelles at low w_0 values, and/or large oligomeric proteins. The stability of large protein aggregates, i.e. of the subunit/subunit interactions is, in fact, still an open question.

As mentioned earlier, the thermodynamic stability of proteins in reverse micelles is necessarily coupled to the question of the structure of the water in the water pool. This is not the only factor affecting stability, although interpretation of the thermodynamic parameters would certainly be rendered more explicit if, for example, we knew the stoichiometry of the water bound to the micelle wall and to the protein. Information on the structure of water cannot come from calorimetric studies. The best methods for this purpose are probably spectroscopic (NMR, IR and similar techniques). Thermodynamic studies must obviously be associated with other types of studies in order to secure an accurate and complete molecular picture.

It is clear that theoretical thermodynamic studies still have a long way to go. The thermodynamic scheme must be coupled with computer processing in order to shed light on the exact relationship which exists between the thermodynamic and structural properties and the micellar parameters. Such experiments are currently underway and are expected to clarify several questions, including that of the change in dimension of the micelle upon uptake of the protein.

Even in its present simplified form, however, the approach of Maestro et al. [40,44] has helped to further our understanding of how the system should be viewed thermodynamically. It has shown that when the protein is incorporated the micellar system assumes a new equilibrium state characterized by a completely new set of parameters, namely the micellar size distribution, the distribution of water in the micelles, the surfactant aggregation number, and so on. In addition, we now have a clearer picture concerning the nature of the driving forces responsible for the uptake of proteins into reverse micelles.

In conclusion we can say that thermodynamic studies on protein-containing reverse micelles, although they are still in their initial stages, have scored

important successes. It can only be hoped that more thermodynamic specialists will turn their attention to this subject and thus create a broader basis for discussion.

ACKNOWLEDGEMENT

We acknowledge with gratitude the financial support of the Swiss National Fund NF19.

LIST OF SYMBOLS

b $(n_a/n_p)(S_a/4\pi r_p^2)$
B $-z^2 + y^2(L+1) + b$
d G/X_a
D $X_a z^2 + X_p - GE + 1$
E $z^3 - X_a l^3 z^2 - X_p j^3 - 1$
f^+ selectivity coefficient for positive tracer ions
f^- selectivity coefficient for negative tracer ions
g r_0/r_p
G $4\pi r_p^3/3v_w$
$\mathcal{G}^o$ $G^o/(NkT)$; reduced free energy
$\mathcal{G}^o_{Ee}$ reduced form of the electrostatic energy contribution to the free energy
$\mathcal{G}^o_{id}$ reduced form of the counterions mixing contribution to the free energy
$\mathcal{G}^o_{mix}$ reduced form of the reverse micelle mixing contribution to the free energy
$\mathcal{G}^o_{Se}$ reduced form of the electrostatic entropy contribution to the free energy
h r_h/r_p
H $[(3y - 2X_a l^3)(3y^2 - 6ly + 4l^2) - 6y(y-l)(y - X_a l^3)]/(y(y - X_a l^3)(3y^2 - 6ly + 4l^2))$
j r_j/r_p
j' value of j corrected for tracer presence
k r_k/r_p
l r_l/r_p
l' value of l corrected for tracer presence
L n_0/n_p
N Avogadro number
n_a surfactant concentration, mol cm^{-3}
n_0 oil concentration, mol cm^{-3}
n_p protein concentration, mol cm^{-3}
n_t tracer concentration, mol cm^{-3}
n_w water concentration, mol cm^{-3}
n_w^0 water concentration in the reference state
N_0 reverse micelles number density before the uptake, $N_0/(n_p N)$
N_e empty reverse micelles number density, $N_e/(n_p N)$
N_f filled reverse micelles number density, $N_f/(n_p N)$

q elementary charge
Q $|q|^2 X_a |z_l| l / 8\pi\epsilon_0 kTr_p$
r_e water core radius of the empty reverse micelles after the uptake (Å)
r_f water core radius of the filled reverse micelles
r_h negative tracer ion radius
r_j protein counterion radius
r_k positive tracer ion radius
r_l surfactant counterion radius
r_0 reverse micelle water core radius before the uptake
r_p protein radius
S_a surface of the surfactant polar head ($Å^2$)
S_p mean protein surface for ionized residue
T absolute temperature (K)
X_a $4\pi r_p^2 |z_a| / S_a |z_l|$
X_p $4\pi r_p^2 |z_p| / S_p |z_j|$
v_p protein volume ($Å^3$)
v_w Water volume ($Å^3$)
V volume of the system
W_0 water/AOT molar ratio
$W_{0,f}$ water/AOT molar ratio in the filled reverse micelle
w $n_a / G n_p$
y r_e / r_p
z r_f / r_p
$|z_a|$ absolute value of the surfactant polar head charge
$|z_h|$ absolute value of the negative tracer ion charge
$|z_j|$ absolute value of the protein counterion charge
$|z_k|$ absolute value of the positive tracer charge
$|z_l|$ absolute value of the surfactant counterion charge
$|z_p|$ absolute value of the mean charge of a protein residue
$|z_j'|$ absolute value of the corrected protein counterion charge
$|z_l'|$ absolute value of the corrected surfactant counterion charge

Greek letters

ΔG° free energy change for the protein inclusion (kcal mol^{-1})
ϵ_0 dielectric permittivity
ϵ_p dielectric constant of the water layer near the protein and at the inner surface of the reverse micelle
σ_p protein surface charge density (q $Å^{-2}$)

REFERENCES

1 M. Zulauf, H.-F. Eicke, J. Phys. Chem., 83 (1979) 480.
2 J.H. Fendler, in Membrane Mimetic Chemistry, Wiley, New York, 1982, Chap. 3.

3 P.L. Luisi and B. Straub (Eds.), in Reverse Micelles, Plenum, New York, 1984.
4 H.-F. Eicke, Top. Curr. Chem., 87 (1980) 85.
5 M. Kahlweit and R. Strey, Angew. Chem., 24 (1985) 654.
6 B. Lindmann and H. Wennerstrom, Top. Curr. Chem., 87 (1980) 1.
7 H.-F. Eicke, J.C.W. Sheperd and A. Steinmann, J. Colloid Interface Sci., 56 (1976) 168.
8 P.D.I. Fletcher, A.M. Howe and B.H. Robinson, J. Chem. Soc., Faraday Trans. I, 83 (1987) 985.
9 B. de Kruijff, P.R. Cullis and A.J. Verkleij, Trends Bioch. Sci., 5 (1980) 79.
10 A.J. Verkleij, Biochim. Biophys. Acta, 779 (1984) 43.
11 M. Wong, and J.K. Thomas, J. Am. Chem. Soc., 98 (1976) 2391.
12 J. Sunamoto, T. Hamada, T. Seto, and S. Yamamoto, Bull. Chem. Soc. Jpn., 53 (1980) 583.
13 P. Douzou, P. Debey and F. Franks, Biochim. Biophys. Acta, 523 (1978) 1.
14 K. Martinek, A.V. Levashov, N.L., Klyachko, and I.V. Berezin, Dokl. Akad. Nauk SSSR, 236 (1977) 920.
15 S. Barbaric and P.L. Luisi, J. Am. Chem. Soc., 103 (1981) 4239.
16 P.L. Luisi and C. Laane, Trends Biotechnol., 4 (1986) 153.
17 P.L. Luisi and L.J. Magid, Crit. Rev. Biochem., 20 (1987) 409.
18 P.L. Luisi, M. Giomini, M.P. Pileni and B.H. Robinson, Biochim. Biophys. Acta, 947 (1988) 209.
19 R. Hilhorst, C. Laane, C. Veeger, FEBS Lett., 159 (1983) 225.
20 P. Lüthi and P.L. Luisi, J. Am. Chem. Soc., 106 (1984) 7285.
21 K.E. Göklen, T.A. Hatton and S.H. Chen, Biotechnol. Progr., 1 (1985) 69.
22 M.E. Leser, G. Wei, M. Maestro and P.L. Luisi, Biochem. Biophys. Res. Commun., 135 (1986) 629.
23 V.E. Imre and P.L. Luisi, Biochem. Biophys. Res. Commun., 107 (1981) 538.
24 G. Häring, P.L. Luisi and F. Meussdörffer, Biochem. Biophys. Res. Commun., 127 (1985) 911.
25 G. Häring and P.L. Luisi, J. Phys., Chem., 90 (1986) 5892.
26 R. Scartazzini and P.L. Luisi, J. Phys. Chem., 92 (1988) 829.
27 W.I. Higuchi and J. Misra, J. Pharm. Sci., 51 (1962) 455.
28 M. Wong, J.K. Thomas and T. Nowak, J. Am. Chem. Soc., 99 (1977) 4730.
29 G. Häring, H. Hauser, A. Pande and P.L. Luisi, J. Phys. Chem., 93 (1989) 7869.
30 C.A. Martin and L.J. Magid, J. Phys. Chem., 85 (1981) 3938.
31 F.M. Menger, G. Saito, G.V. Sanzero and J.R. Dodd, J. Am. Chem. Soc., 97 (1975) 909.
32 R. Wolf and P.L. Luisi, Biochem. Biophys. Res. Commun., 89 (1981) 209.
33 F.M. Menger and K. Yamada, J. Am. Chem. Soc., 101 (1979) 6731.
34 K. Martinek, Y.L. Khmelnitsky, A.V. Levashov, N.L. Klyachko, A.N. Smenov and I.V. Berezin, Dokl. Akad. Nauk SSSR, 256 (1981) 1423.
35 E.A. Malakhova, B.I. Kurganov, A.V. Levashov, I.V. Berezin, and K. Martinek, Dokl. Akad. Nauk SSSR, 270 (1983) 474.
36 E. Battistel, P.L. Luisi and G. Rialdi, J. Phys. Chem., in press.
37 Y. Fujita and Y. Noda, Bull. Chem. Soc. Jpn., 57 (1984) 2177.
38 J.F. Brandts, J. Am. Chem. Soc., 86 (1964) 4291.
39 J.F. Brandts and L. Hunt, J. Am. Chem. Soc., 89 (1967) 4826.
40 M. Caselli, P.L. Luisi, M. Maestro and R. Roselli, J. Phys. Chem., 92 (1988) 3899.
41 L.M. Gierasch, K.F. Thompson, J.E. Lacy and A.L. Rockwell, in P.L. Luisi and B.E. Straub, (Eds.), Reverse Micelles, Plenum, New York, 1984, p. 21.
42 C. Petit, P. Brochette and M.P. Pileni, J. Phys. Chem., 90 (1986) 6517.
43 C. Grandi, R.E. Smith and P.L. Luisi, J. Biol. Chem., 156 (1981) 837.
44 M. Maestro and P.L. Luisi, Proc. 6th Int. Symp. on Surfactants in Solution, New Delhi, August 18–22, 1986, in press.