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Proteins as Invited Guests of Reverse Micelles: Conformational Effects, Significance, Applications

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Abbreviations: ACTH, adrenocorticotrophic hormone; AOT, aerosol-OT; ARMES, affinity-based reverse micellar extraction and separation; CD, circular dichroism, CTAB, cetyl trimethylammonium bromide; cyt c, cytochrome c; D_2O, deuterated water; FTIR, Fourier transform infra red; HPLC, high performance liquid chromatography; HRP, horse radish peroxidase; HSA, human serum albumin; MBP, myelin basic protein; NAG, N-acetyl glucosamine; NATA, n-acetyl tryptophanamide; SBP, soybean peroxidase; TPI, triose phosphate isomerase.

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Introduction

Among the many specific and non-specific interactions which play key roles in stabilizing the structure of proteins, the presence of water of hydration, although an often neglected factor, seems of particular importance. Water relations are evidently crucial in many biological processes (Finney *et al.*, 1980) including enzymatic reactions, ligand recognition, specific (antigen-antibody) or non-specific macromolecular associations and oligomerizations. In the last decades a common practice among protein chemists or biochemists has been to vary two or at most three experimental parameters in the course of their investigations: the concentration of the reactants, the temperature during which the reaction develops and time elapsed in kinetic measurements. Other parameters, more difficult to control, have been less taken into consideration, be it the number and the nature of water molecules participating directly or not to the reaction, or the space allocated to shelter the bioactive macromolecule.

Space is obviously limited inside a cell, inducing confinement conditions which may alter the chemical potential of molecules (Minton, 1992, 1995). There is now firm evidence that the physico-chemical microenvironment existing within the cell is significantly different from the milieu where many biochemical experiments are carried out, *i.e.* dilute aqueous solutions. As pointed out by Parsegian *et al.*, (1986): 'The many kinds of association and dissociation and of assembly and disassembly that go on inside a cell do not occur in the dilute solution where they are usually studied, but rather under conditions of exquisitely controlled hydration and competition for aqueous solvent.'

In this context it is important to recall that many biological events of fundamental importance take place at membrane interfaces, in compartments where both water and space are scarce. As a consequence during the last decade, membrane-mimetic agents (Fendler, 1982) have been a growing area of research directed to the exploitation of membrane-mediated processes in relatively simple chemical systems. While these basic molecular assemblies lack many of the complex features of their biological counterparts, a number of model systems have preserved several specific properties of native membranes, as well as the functionally important interface between surfactant/lipid headgroups and sequestered water. These systems constitute one aspect of organized assemblies which include monolayers, bilayers, micelles, and vesicles.

Among them water-in-oil microemulsions or reverse micelles attracted the attention of physicists of colloidal systems (soft matter) as early as 1943 (Hoar and Schulman), and later (Mathews and Hirschhorn, 1953). They have been described as water microdroplets, dispersed in apolar solvents and stabilized by a monolayer of surfactant. As shown in *Figure 1*, the water is surrounded by the polar headgroups of

the surfactant whose non-polar tails protrude outward into the organic solvent. Reverse micelles, or inverted micelles, are inside out with respect to more common micelles formed from surfactants dissolved in water. The size of these spheroidal microaggregates, which are thermodynamically stable, depends solely on the molar ratio of water-to-surfactant (Wo). They allow a precise experimental control of space and water available inside the micelle. Reverse micelles of protein size (in a scale of nanometers) are optically transparent. Fundamental dynamics of the system (Fletcher, Howe and Robinson, 1987) involve processes of rapid collision (on a time scale of nanoseconds) and exchange (in a few microseconds) of solubilized material and allow reagents and macromolecules to come in contact and to react.

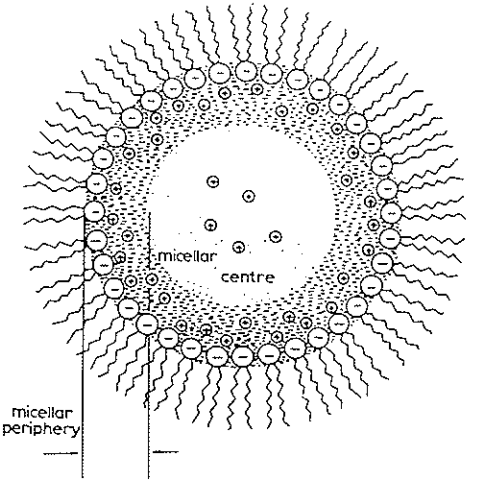

Figure 1. Scheme of a reverse micelle. At the periphery, the surfactant polar head groups are in contact with bound water (in equilibrium with central bulk water and counterions), while the hydrocarbon tails are in contact with the organic solvent and the tails of neighbouring micelles. (Luisi *et al.*, 1988 with permission.)

Very soon it became obvious to investigators that the interior of reverse micelles *i.e.* the micellar inner interface and the aqueous phase, could provide a unique and versatile reaction field for the study of biocatalyst transformation, by housing guest enzymes together with substrates (Martinek *et al.*, 1977) into these organized microassemblies. At the same time, Wolf and Luisi (1979) published a structural study of ribonuclease confined in reverse micelles and reached the conclusion that the conformational features of the enzyme, as judged from circular dichroism spectra, closely resembled those measured in aqueous solutions. This statement may still hold for a number of proteins, but contrary to expectations reality proved again to be more complex than anticipated (Luisi and Magid, 1986).

One of the major problems encountered by investigators was that conformational studies require high protein concentration in order to obtain reliable data as compared to enzyme kinetics, an objective sometimes difficult to achieve in micellar solutions, due to the variability of protein solubilities. The same applies for the exploration of fundamental protein mechanisms such as folding-unfolding transitions, protein-

ligand binding or protein-protein interactions. As a direct consequence, a great number of papers dealing with protein folding or the state of enzyme aggregation have avoided display to structural parameters and have relied rather on enzyme kinetic data obtained at low protein concentration (*i.e.* very low protein occupancy of the micelles initially present). Accordingly there is only limited information concerning conformational issues in reverse micelles. Questions relevant to properties of proteins in water-restricted environments were addressed earlier in the last decade (Levinthal, 1986; Waks, 1986). Nonetheless, the literature has kept growing, bringing to light a number of novel results and significant advances in the field.

The physical and chemical properties of the system as well as catalytic characteristics of enzymes in reverse micelles were reviewed last year in detail by Oldfield (1994). The primary focus of this review is, rather, a critical analysis of the significant events occurring after solubilization of biological macromolecules. Our intention is to avoid overlapping of these reviews, although it will be difficult at times. It is clear however, that new generation biotechnologies demand a detailed analysis of the mechanisms affecting structural alterations in proteins (or protein domains) hosted in reverse micelles. Accordingly we shall adopt a somewhat different perspective by electing a protein-guest, rather than a micelle-host point of view as has more generally been done (Luisi, 1985; Luisi *et al.*, 1988). First we will describe the intricate microenvironment surrounding the protein after incorporation into micelles. We will try then to obtain insight into the origin of changes induced in several passenger proteins or enzymes with an emphasis on well identified conformational processes. We will also turn to functionally important protein-protein interactions like those involving molecular recognition, and enzyme self-association or oligomerization mechanisms. Finally we will review a few recent and innovative biotechnological applications which point to interesting directions for the future.

The protein microenvironment inside the reverse micelle

Within the aqueous microdroplet a subtle interplay occurs between the micellar wall, encapsulated water and the protein. These interactions have been documented essentially for micelles of the anionic surfactant, bis (2-ethylhexyl) sodium sulfosuccinate (AOT). As far as water is concerned, the negative AOT sulfonate groups interact with water molecules coordinated to hexahydrated sodium ions, which are in turn hydrogen bonded to the polar groups of the surfactant molecule, generating thus the bound water layer (Maitra, 1984). A Fourier-Transform Infrared (FTIR) study by Christopher *et al.*, (1992) revealed that the first three water molecules are strongly bonded to the anion but that this interaction also involves the cation Na^+. Addition of three more water molecules results in a fully hydrated cation. In fact, Hauser *et al.*, (1989) have observed using ^2H NMR spectroscopy, that the water environment remains highly perturbed up to a ratio of 12 water molecules per surfactant head group. Using differential scanning calorimetry, they also showed that 6 water molecules from the hydration shell of AOT-Na^+ are perturbed so much that freezing is prevented at subzero temperatures. In addition, strong ion-dipole and dipole-dipole interactions exist between protons and AOT polar head groups. Such a situation leads to an overall disruption of the tetrahedral arrangement of water molecules that maintains the low

packing density characteristic of the open lattice-like structure in the unperturbed solvent.

The latter phenomenon probably accounts for the collapse of the specific volume of water at micellar interfaces (Day *et al.*, 1979; Scherer, 1987, White, Jacobs and King, 1987; Etchells and Kahn, 1993; Kahn, Urbach and Waks, 1993) and for the anomalous variation of adiabatic compressibility of AOT micellar solutions as a function of the water content of the system (Amarene *et al.*, 1995). It has been suggested that hydrogen bonding between the polar head groups and water may constitute the ordering potential for the hydration layer (Gawrisch *et al.*, 1992)

At low water-to-surfactant molar ratios, competition for essential water (one or two water layers around the macromolecule) takes place between the surfactant polar head groups and the dry protein molecules introduced into reverse micelles. A striking example of competition for water is illustrated in *Figure 2A*, which depicts the solubilisation in reverse micelles of Myelin Basic Protein (MBP), an extrinsic membrane protein: it displays a very unusual pattern. While in many water-soluble proteins solubility increases in reverse micelles with Wo within a wide range of water, oil and surfactant concentrations (Grandi, Smith and Luisi, (1981), the solubility of MBP (M.W.=18 500) rises sharply until a maximum located around Wo=5.5 and then drops at higher Wo values. Since at Wo=5.5, all the water present is tightly bound to surfactant polar head groups and the radius of the micellar water core is smaller than the hydrodynamic radius of MBP, a different solubilization mechanism must be involved. Indeed, Chatenay *et al.*, (1985) have shown by dynamic light scattering experiments that the protein 'picks up' about three smaller-sized micelles to build up a larger tailor-made shelter.

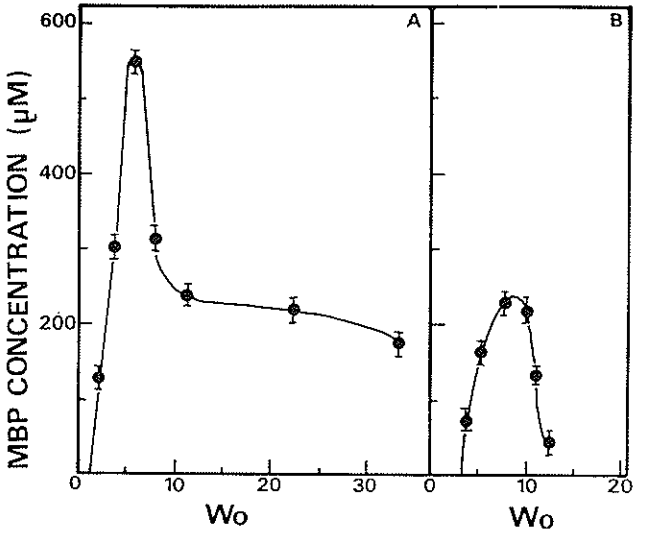

Figure 2. Solubilisation curve of MBP in reverse micelles of surfactant in isooctane and water. The overall protein concentration is measured from the final absorbance at 278nm. In curve A the surfactant is 0.05 M AOT and in curve B, 0.1 M tetraethylene glycol monododecyl ether (Nikkol). (Nicot *et al.*, 1985) Bars represent experimental error.

In the latter case it is obvious that the water-soluble protein 'prefers' to be housed in the tailor-to-measure micelle rather than in a ready-made, protein-size micelle (Wo=12). This result has been interpreted by Nicot *et al.*, (1985) as arising from the high affinity of the protein for interfacial water at the membrane-mimetic interface. Such water possesses many physicochemical properties of micellar water originating from the intense electrostatic field of the ionic groups. In contrast, the solubility curve of MBP carried out in a non-ionic surfactant shows that the total amount of protein solubilised drops and the maximum of solubilisation is considerably shifted to higher Wo values (*Figure 2B*). Leser *et al.*, (1986) have confirmed this type of behavior with a basic enzyme, lysozyme, (M.W.=14 400) which displays an optimal solubility at a Wo value near 7.0 designated by the authors as 'micellar solubility'. Incidentally, these results appear to contradict the mechanism of protein solubilization proposed by Matzke *et al.*, (1992), since in both cases the optimal solubility does not occur in the presence of protein-sized micelles but rather of much smaller ones.

The dielectric constant of bound water is another parameter to consider as a possible factor inducing conformational changes. There is general agreement that at Wo values below 10, the dielectric constant and polarity of the encapsulated water is much lower than that of bulk water (Kommaredi, O'Connor and John, 1994), but precise experimental values are difficult to obtain. The main difficulty arises from the nature of the probes used for such determinations, which, depending on their chemical structure and amphiphilic character, can be located alternatively within the aqueous core, at the interface or even within the organic solvent, where values of dielectric constant may differ considerably. A low dielectric constant will also enhance the strength of hydrogen and electrostatic bonds (Herron *et al.*, 1994), which can profoundly influence protein structure, since electrostatic interactions have destabilizing effects and favor the unfolded states of macromolecules (Honig and Nicholls, 1995). Alternatively we will see that, depending upon circumstances, electrostatic interactions may be also able to refold several basic biological peptides.

Another consequence is that the dynamic fluctuation of the protein will decrease in reverse micelles, the less mobile residues being located near the protein exterior (Affleck, Haynes and Clark, 1992; Affleck *et al.*, 1994). The macromolecular motion inside the micelles will be further hindered by the high microviscosity of the water pool which was found to be equivalent to a 78% glycerol solution (Tsujii, Sunamoto and Fendler, 1983) or even to still higher values (Hasegawa *et al.*, 1994), at low Wo values. Overall such effects may lead to the so-called 'frozen structures' described in a number of proteins in buffer or in reverse micelles (Waks and Beychok, 1974; Walde *et al.*, 1988; Klyachko *et al.*, 1989; Dorovska-Taran, Veeger and Visser, 1993b).

Assertions that a frozen conformation corresponds to the most active one in reverse micelles (Khmelnitzky *et al.*, 1989), which may occur when the radius of the inner aqueous cavity of the micelle is very close to the size of the enzyme, have hardly been substantiated by experimental data. On the contrary, different authors suggest that enzymes require enough conformational freedom to exhibit measurable catalytic activity (Ayala *et al.*, 1992; Rasmussen *et al.*, 1992). Strambini and Gonelli (1988) have attributed the results of phosphorescence studies on liver alcohol dehydrogenase in reverse micelles, to a greater flexibility of the coenzyme binding domain. From fluorescence anisotropy decay measurements Visser *et al.*, (1994) have shown that the single tryptophan (at postion 140) of staphylococcal nuclease, a small, monomeric,

reversibly folding protein (Shortle and Meeker, 1986), rigid on a nanosecond scale in buffer, acquires an additional internal flexibility in reverse micelles. These issues remain still to be explored.

EXPERIMENTAL COMMENTS

Since protein-sized reverse micelles are optically transparent, they allow the use of all the tools of solution biochemistry in particular spectroscopy. From a practical point of view, the solubilisation of proteins is in general performed without major difficulties by addition of carefully measured volumes of an aqueous solution of protein to a solution of the surfactant in organic solvent. When larger amounts of protein are needed, dried, preweighed quantities of protein are added to preformed micellar solutions containing the desired amount of water; caution should be exercised to avoid an excess of solid protein which precipitates taking up water and surfactant (Nicot *et al.*, 1985). The right amount of soluble protein can be previously determined by trial and error and checked by absorbance measurements. Membrane proteins which are water insoluble (or scarcely soluble proteins) can be incorporated into preformed micelles after precipitation by the same organic solvent that was used for the micellar solution (for more details see Vacher, Waks and Nicot, 1989; Groome *et al.*, 1990).

For the investigation of conformational changes, static and time-resolved fluorescence studies have been carried out, in particular on single tryptophan macromolecules. In a number of proteins and peptides studied by steady-state fluorescence, a significant blue shift (from 3 to 15 nmm) in the maximum emission spectrum has been observed in micellar solutions, after tryptophan excitation at 290nm. The blue shift decreases gradually with increased Wo values, leading to a plateau value around 350 nm for a fully exposed tryptophan, as measured in N-acetyl tryptophanamide (NATA). The final value is always slightly lower than that recorded in bulk water but the reason is not clear (Grandi, Smith and Luisi, 1981; Nicot *et al.*, 1985; Gallay *et al.*, 1987 Marzola and Gratton, 1991).

The blue shift originates from a combination of the low polarity, low dielectric constant and high viscosity of the microenvironment within the micellar aqueous pool. Alternatively it may be attributed to possible conformational changes which bury the tryptophan into a newly folded protein domain, rendering it inaccessible to the solvent. It has also been suggested that the blue shift might also reflect modification of hydrogen bonding between the indole and the structured micellar water (Ferreira and Gratton, 1990). Fluorescence decays are mostly rather complex. Time-resolved polarization spectroscopy is well documented now, and has brought interesting information concerning fluorescent species in solution by measuring their rotational correlation times which are directly related to the hydrodynamic radius of the spherical micelles. Nonetheless, these techniques cannot alone provide direct access to protein conformational changes occurring in reverse micelles and therefore require confirmation by more definitive techniques.

A number of measurements have been therefore carried out using far ultraviolet (250–200 nm) circular dichroism (CD) involving the peptide chromophore, which allows determination of both conformation and change in conformation of proteins and peptides. As in buffer, the near-ultraviolet spectrum due to the many overlapping bands (Kahn, 1979) (tryptophan, tyrosine, phenylalanine, disulfide), is frequently

more difficult to interpret since it detects essentially changes in the environment of aromatic chromophores.

One difficulty to overcome in spectral measurements arises from the absorbance of the surfactant AOT which starts at 260nm, increases at shorter wavelengths and results in an unfavourable signal-to-noise ratio (Luisi *et al.*, 1984). It can be compensated to some extent by the use of extremly short CD cells or by working at low surfactant concentration. It is worth noting that nonionic surfactants such as poly(oxyethylene) ethers, belonging to the C_iE_j family (Nikkol), display a very low specific absorption coefficient down to 200nm. They can be used at high concentration and are well suited for CD measurements in reverse micelles. In addition they are devoid of the strong electrostatic interactions which may impair the conformation and the catalytic activity of several positively charged enzymes, as will be seen below.

Conformational effects in reverse micelles

FOLDING OF PROTEIN STRUCTURES

Several investigators have reported the folding of proteins or peptides in reverse micelles (Nicot *et al.*, 1985, Gallay *et al.*, 1987; Hagen, Hatton and Wang, 1990a, b) although such experiments were not initially directed to the study of the folding process *per se*. The work of Hagen and co-workers (1990a, b) for example, was undertaken to circumvent problems of aggregation reducing the yield of recombinant proteins obtained from bacterial inclusion bodies. It requires prior dissociation and unfolding in denaturant solution before refolding. In a different set of experiments, a peptide displaying initially little periodic structure in aqueous solvents, was observed to interact and refold at the micelle inner wall, as a mimic of a membrane or receptor-like interface (Gallay *et al.*, 1987), or perhaps adopt molten-globule structures like insulin in reverse micelles (Lenz *et al.*, 1995). A number of fundamental questions arise from the available literature that have not yet been satisfactorily answered. For example, in the absence of any structural information can we infer the refolding of an enzyme into its native conformation solely from recovery of its enzymatic activity? Conversely what are the folding/unfolding mechanisms involved? To what extent a folded structure can remain stable in reverse micelles? Finally, what will we learn about protein structure, dynamics or function from a conformational transition observed in reverse micelles?

Myelin basic protein

The most dramatic example of protein refolding in reverse micelles was reported by Nicot *et al.* (1985). Myelin basic protein (MBP) is a protein of the highly specialized multilamellar myelin membrane from the central nervous system. The protein has no disulfide bridge, and a single tryptophan. Although its three-dimensional structure within the myelin sheath is unknown, there is general agreement that in dilute aqueous solutions it displays a high degree of conformational flexibility, with possible locally ordered areas (Lees and Brostoff, 1984). Stoner (1984) and Martenson (1986) have modelled the organization of hydrophobic segments into a beta-sheet in myelin. Upon incorporation into reverse micelles, MBP acquires a more ordered, compact structure

(*Figure 3*), as anticipated in native myelin, displaying about 20% alpha-helix, together with beta-turns and beta-bends (Nicot *et al.*, 1985). Furthermore, the same authors showed that the new periodic protein structure was stable at Wo values from 2.0 to 22.4 and therefore independent of the amount of water present in the system. The 'micellar' folded conformation of MBP was subsequently confirmed by Surewicz, Moscarello and Mantsch , (1987) by FTIR spectroscopy in negatively charged vesicles of synthetic phospholipids.

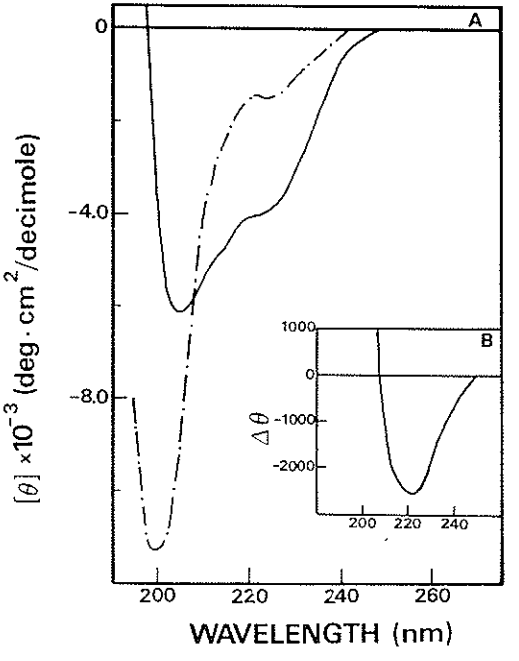

Figure 3. Circular dichroism spectrum of MBP. A. Far-ultraviolet CD of the protein in aqueous (— · —) and in micellar solution (——) in 0.05M AOT in isooctane and water at Wo=5.6. B. Calculated difference spectrum between spectra measured in aqueous and micellar solutions. (Nicot *et al.*, 1985.)

One of the important issues addressed by these results is the following: to what extent are the observed conformational changes induced by specific interactions between the protein and the micelle constituents? Unlike alpha-globin for example (Iyer and Acharya, 1987), addition of 1-propanol does not cause MBP to refold in buffer into an ordered, periodic structure (Nicot *et al.*, 1993). Furthermore, reverse micelles of a non-ionic surfactant tetraethylene glycol monododecyl ether, were unable to generate the same fraction of alpha-helix in the protein (Nicot *et al.*, 1985).

Although no model system can faithfully mimic the complex architecture of the native myelin membrane, it has been shown that the interlamellar aqueous spaces of myelin share a number of features with AOT reverse micelles at Wo values below 7.0, (Nicot and Waks, 1989). In brief, in both organized structures the affinity of charged polar head groups for water is the dominant force opposing close apposition of the surfaces. The high degree of organization of the water measured by dielectric studies of myelin (Gent, Grant and Tucker, 1970) is comparable to the water of reduced

mobility present in reverse micelles. Finally the distances between the apposed lipid or surfactant head groups are of comparable magnitude (30–40 Å).

These organized microassemblies thus appear to preserve much of the amphipathic and the anisotropic nature of the biological membrane interface. Such a heterogeneous physicochemical environment includes charge density gradients, the availability of hydrogen bonding groups, local proton activity, and polarity (Gawrisch *et al.*, 1992), in addition to the dielectric gradients at the interfacial region (Leodidis and Hatton, 1989) and hydration forces (Israelshvili and McGuiggan, 1988; Rand and Parsegian, 1989). In conclusion, the conformation of MBP observed in reverse micelles is not therefore the result of just another cosolvent-assisted refolding process (Cleland and Wang, 1990), but appears specific in nature and requires the subtle interplay of forces operative in both AOT reverse micelles and in the interlamellar aqueous spaces of native myelin (Nicot and Waks, 1989; Nicot *et al.*, 1993).

The Folch-Pi myelin proteolipid.

Although micellar solutions seem to provide an ideal milieu for solubilization of membrane proteins, the Folch-Pi proteolipid (a lipid-protein complex) the major protein from myelin membrane was the first and probably the sole integral membrane protein inserted so far in reverse micelles in a stable conformation (Delahodde *et al.*, 1984). The proteolipid, totally insoluble in water, was previously solubilized and studied in aqueous detergent solutions or in organic solvent mixtures such as chloroform-methanol (Lees and Brostoff, 1984). It was therefore unexpected to find that the solubilization process in reverse micelles required a small but definite number of water molecules (between 6 and 7). Further studies revealed that more than one micelle per macromolecule was involved in the solubilization mechanism (Delahodde *et al.*, 1984). In addition, 6 to 10 tightly bound lipids which increased its hydrophobic character, were also a prerequisite for the incorporation of the protein into the membrane-mimetic system (Vacher, Waks and Nicot, 1989).

The conformation of the protein (M.W.=30 000) in the native myelin is not known, but in reverse micelles structural studies carried out by CD in the far-ultraviolet (*Figure 4*), show that the proteolipid is highly helical (55% of alpha-helix). This is much less than previously reported by Cockle *et al.*, (1978) in 2-chloroethanol (75%). However, it is widely known that organic solvents can induce an artificially high helical content in proteins (Singer, 1962; Jackson and Mantsch, 1992; Shiraki, Nishikawa and Goto, 1995). Thus it is not unreasonable to expect that the proteolipid displays much less periodic structure in reverse micelles, than in mixtures of organic solvents where it has been studied previously.

From the most recent models of the proteolipid obtained from sequence and hydrolytic studies (four hydrophobic alpha-helices with 6 covalently bound fatty acids penetrating the bilayer,) it appears that the protein contains only 55% of alpha-helix (Weimbs and Stoffel, 1992). The structure confirmed subsequently in a synthetic phospholipid environment by FTIR spectroscopy (Surewicz, Moscarello and Mantsch, 1987) is in good agreemnt with the results of Delahodde *et al.*, (1984) in AOT reverse micelles. Because of the similarity of the physicochemical forces existing in the aqueous spaces located between myelin bilayers and the water of reverse micelles

(Nicot *et al.*, 1985), it is conceivable that interactions among the protein, water and the polar head groups may favor a conformation, possibly pre-existing in myelin lamella which confers on the protein its solubility properties in the membrane-mimetic system.

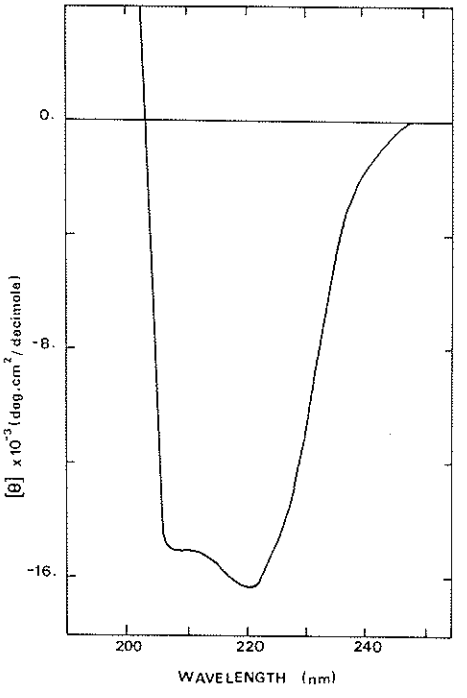

Figure 4. Far-ultraviolet CD spectrum of the Folch-Pi proteolipid in AOT (from 0.05 to 0.150 M) reverse micelles, in isooctane and water at a Wo value of 5.6. The spectrum is independent of surfactant concentration. The ellipticities are expressed on a mean residue basis (Delahodde *et al.*, 1984.)

Recently, the myelin proteolipid has been also solubilized in reverse micelles of the non-ionic surfactant tetraethylene glycol monododecyl ether (Merdas *et al.*, 1995). The conformation of the protein is identical to that found in AOT reverse micelles, illustrating the stability of the protein structure in the biomimetic environment and providing further insights on the solubilisation mechanism. In contrast to its companion myelin basic protein (MBP), a water-soluble protein, positive charges of the polar proteolipid domains are not required either for the solubilisation process or for the preservation of the protein three-dimensional architecture. These findings are supported by the data reported by Jones and Gierash (1994) concerning the signal peptide-lipid interactions for the *Escherichia coli* LambB signal sequence. The authors have shown by tryptophan fluorescence spectroscopy, that the driving force for the insertion of the signal peptide into the membrane is mainly hydrophobic. It seems thus obvious that mechanisms responsible for the insertion of proteins in reverse micelles are closely related to the specific sequence and structure of the protein but also to the membrane-mimetic milieu.

Rhizopus arrhizus *lipase*

Lipases are enzymes activated by interfaces (Verger, 1980) and their behavior in reverse micelles is of special interest. The structure measured in near and far-ultraviolet CD and the lipolytic activity of the *Rhizopus arrhizus* lipase have been examined by Brown, Yada and Marangoni (1993). The enzyme, soluble in aqueous solvents, exhibits an alpha-helical structure and very little beta-sheet. Incorporation into reverse micelles induces only a modest increase of alpha-helix (from 10% to 12%) but a substantial increase in beta-sheet structure (from 1.1% to 24%) as well as in beta-turns (from 4% to 16%). At the same time, a fine structure appears in the near-ultraviolet (*Figure 5*), due either to a protein reorganization or to the interaction of the aromatic amino acids with parts of the micellar interface. The spectral changes are paralleled by a 10 nm blue shift in the fluorescence emission maximum wavelength. A similar observation has been also reported by Marangoni (1993) for porcine pancreatic lipase, in AOT reverse micelles. At Wo=11.85, in 0.1M Tris-HCl buffer pH 9.0, a substantial increase of beta-sheet (around 30%) is measured by far-ultraviolet CD, accompanied by a loss of alpha-helix (*Figure 6*).

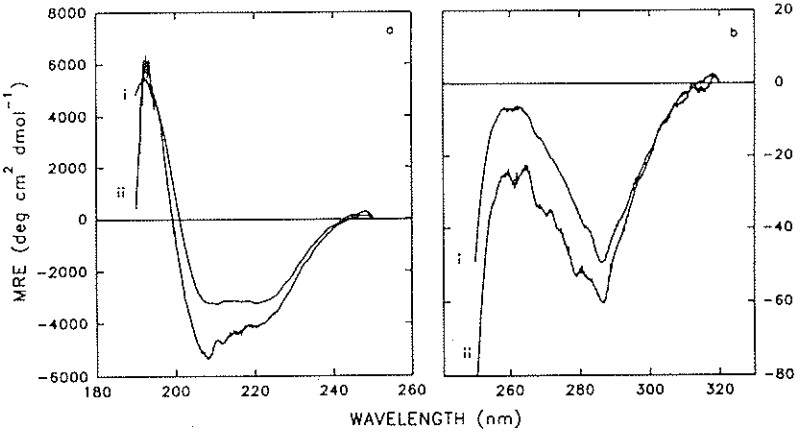

Figure 5. Circular dichroism spectrum of *Rhizopus arrhizus* lipase. a: Far-ultraviolet region (i) in 0.05 M sodium phosphate, pH 7.4 and (ii) in reverse micelles of 0.05 M AOT in isooctane, Wo=11 in the same buffer. b: near-ultraviolet spectrum (same conditions as in a) MRE: mean residue ellipticity. (Brown, Yada and Marangoni, 1993, with permission.)

The authors suggest that a mechanism resembling that elucidated by Brzozowski *et al.*, 1991, and Derewenda *et al.*, 1992 for *Rhizomucor miehei* lipase by X-ray crystal analysis in the presence of an inhibitor, is also operative in *Rhizopus arrhizus* and in porcine lipase. The structural reorganization observed in both lipases upon micellar incorporation might thus be due to the movement of an alpha-helical hydrophobic lid uncovering the active site of the enzyme and expanding the hydrophobicity of the area surrounding the catalytic site. Interfacial activation would thus originate from stabilization of the nonpolar surface by the lipid or surfactant environment. Nevertheless the extension of these patterns to all lipases in reverse micelles, as suggested by Marangoni (1993), requires caution.

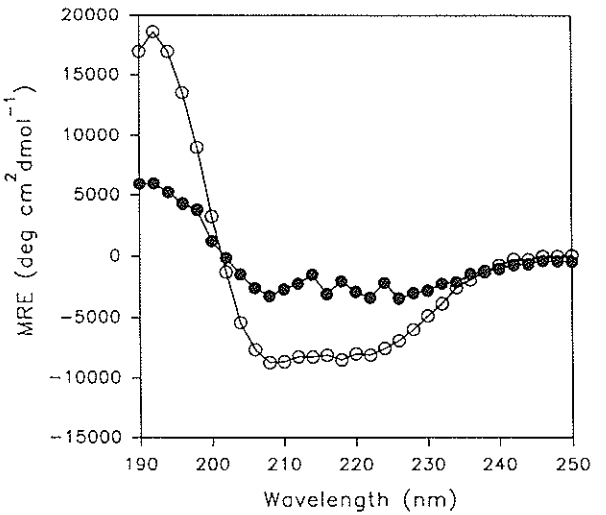

Figure 6. Far-ultraviolet CD spectrum of porcine pancreatic lipase in 0.1 M Tris-HCl buffer, pH 9.0 (OOOO) and in reverse micelles of 0.05 M AOT in isooctane (●●●), at Wo=11.85 with the same buffer. (Marangoni, 1993, with permission.)

Triosephosphate isomerase

The refolding of triosephosphate isomerase (TPI), a homodimeric enzyme previously dissociated into monomers, has been investigated in reverse micelles formed by a cationic surfactant cetyl trimethyl ammonium bromide (CTAB), n-octane, hexanol and water. Fernandez-Velasco *et al.*, (1995) have investigated the precise amount of water required for subunit refolding after dissociation and denaturation by guanidine hydrochloride. By recording the intrinsic fluorescence emission spectra of TPI, they observed that transfer of the enzyme into micelles in the presence of 2% water yielded monomers that were unable to dimerize and therefore lacked catalytic activity. In contrast, micelles containing as much as 5% of water were able to trap monomers competent for further dimerization. Thus the dimerization mechanism requires in addition to the association between monomers, a refolding process taking place in sufficient water to fill defined physical limits of the micelle. One important question which remains unanswered is whether at 5% of water there is space enough for dimers or only for monomers, but it appears that the authors did not measure the micellar size at any water content.

Peptides bioactive at interfaces

The general wisdom is that peptides are convenient models for protein domain folding/unfolding studies. Moreover, since the influence of a micellar environment is distinct from any isotropic bulk phase, reverse micelles provide an excellent model to explore the conformational impact of an interface on a peptide. A number of them display a highly flexible conformation in aqueous solutions and several contain a

single tryptophan residue which serves as a sensitive fluorescent probe, reflecting the dynamic properties of its microenvironment. Investigation of peptide secondary structure supports the proposal that the structure induced in reverse micelles is not an artifact of the environment, but rather an indication of the conformation that the molecule adopts when in close proximity to a membrane surface and possibly when bound to a receptor.

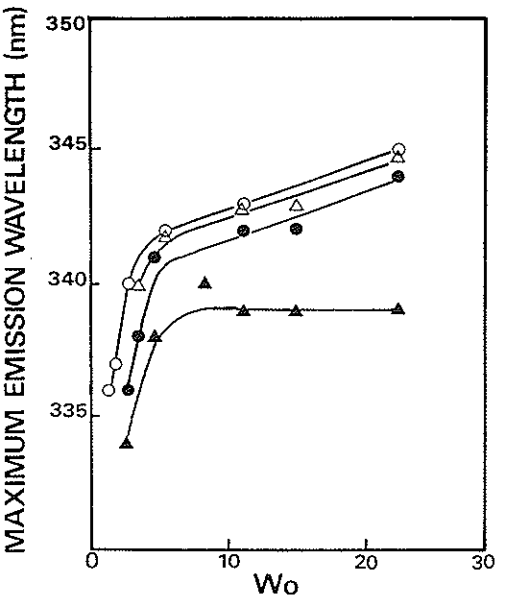

Figure 7. Variation of the maximum fluorescence emission wavelength of ACTH (1–24) (▲▲▲), glucagon (●●), ACTH (5–10) (ΔΔ) and NATA (OOO) as a function of the water-to-surfactant molar ratio (Wo) in reverse micelles of 0.05 M AOT and water in isooctane. (Gallay *et al.*, 1987.)

A number of peptides active *in vivo* at membrane interfaces have been solubilized in reverse micelles. Gallay *et al.* (1987) using steady state and time resolved fluorescence spectroscopy, have explored in detail the respective importance of charges and of water structure in the conformational changes of synthetic adrenocorticotropin peptides ACTH(1–24) and (5–10). The experiments were carried out in AOT reverse micelles, at various Wo values and by comparison with a small fluorescent molecule, N-acetyl-tryptophanamide used as a probe. While for NATA and ACTH(5–10) the wavelength of maximum fluorescence emission varies continuously as a function of the physical properties of entrapped water (*Figure 7*), ACTH(1–24), with 6 positive charges in its sequence, displays a variation in the emission maximum from 334nm at Wo=2.0 to 338nm at Wo=7.0. This result indicates that the chromophore is shielded from water by the attractive peptide interactions with the micellar inner wall. At the same time ACTH(1–24) undergoes a dramatic conformational change from beta-sheet (at Wo=2) to an unfolded structure at high water content (Wo=30) illustrating the interactions between the peptide backbone and the mobile water dipoles (*Figure 8*). The reaction is reversible and indicates that the electrostatic bonds alone fail to prevent unfolding of a peptide when the amount of micellar water is adequately increased.

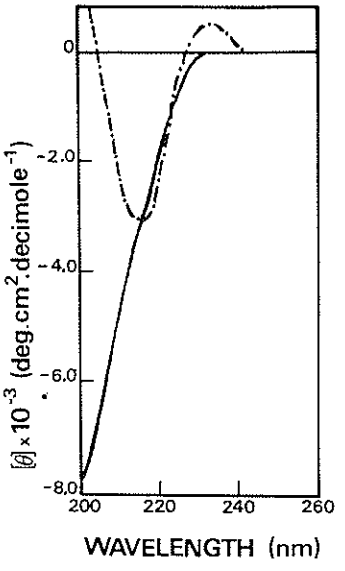

Figure 8. Far-ultraviolet CD spectrum of ACTH (1–24) in reverse micelles of 0.025 M AOT and water, at Wo values of 3.5 (· — · — ·) and 22.4 (——) (Gallay *et al.*, 1987.)

By contrast, glucagon displays a very different picture. Whereas in dilute aqueous solutions it exhibits large amounts of extended, flexible structure, incorporation of the hormone in reverse micelles at low Wo values induces a significant contribution of alpha-helix (*Figure 9*). The ordered structure is unaffected by the amount of water present. These results are in agreement with structural information obtained from ^{1}HNMR measurements carried out on glucagon at a lipid/water interface by Braun *et al.* (1983). The alpha-helices, once folded, form a cluster of several hydrophobic amino acids which are not prone to be disrupted by free water. Guz and Wasylewski (1994) have reported a similar pattern for the folding to an alpha-helical conformation of mellitin, a 26 amino acid peptide from bee venom, in AOT reverse micelles. Here again the peptide structure once folded at the membrane-mimetic interface, is unaffected by increasing the water content of micelles, revealing thus the stabilization of a peptide conformation otherwise disordered in buffer (*Figure 10*). It is interesting to mention in this regard that mellitin, a pore forming peptide in lipid bilayers, adopts a transmembrane topology when dehydrated, whereas in presence of water it binds to the micellar surface (Smith *et al.*, 1994).

There are other reports in the literature examining the behavior of bioactive peptides in reverse micelles. Lenz *et al.* (1995) have studied the conformation of insulins labelled with 2-aminobenzoic acid, in AOT reverse micelles. The hormone preserves its folding in the far-ultraviolet CD, but from the weak intensity of circular dichroism bands in the near-ultraviolet, the authors claim that insulin behaves as a molten globule (Ptitsyn, 1987; Christensen and Pain, 1991) in AOT reverse micelles. Further studies are required to test this hypothesis, if as proposed by Hua, Ladbury and Weiss, (1993), the functional form of insulin is indeed a molten globule.

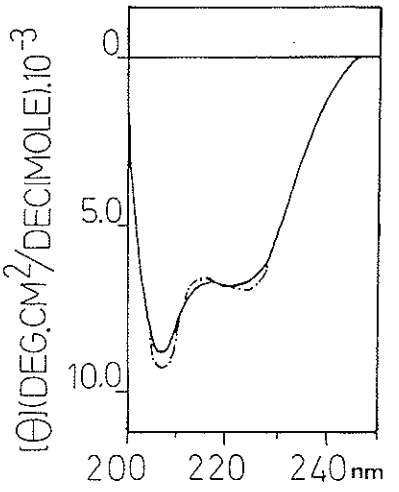

Figure 9. Far-ultraviolet CD spectrum of glucagon in reverse micelles of 0.025 M AOT and water. Wo values were 3.5 (——) and 22.4 (· — · —) (Gallay *et al.*, 1987.)

The alpha-melanocyte stimulating hormone which contains the first thirteen amino acids from the sequence of ACTH (with which it shares a number of biological properties) has been studied by Bhattacharyya and Basak (1993) using time-resolved fluorescence and quenching dynamics. Unfortunately the study is mainly devoted to the localization of the peptide in the micelle, near the AOT-water interface. The same authors have also investigated another peptide hormone, somatostatin (Bhattacharyya and Basak, 1995) using circular dichroism in addition to fluorescence spectroscopy. They report that in water the peptide displays beta-turn structures, the amount of which seems to increase in reverse micelles. A quantitative estimation is, however, difficult in short peptides because of the additive contributions to the CD of aromatic amino acids as well as disulfides in the far-ultraviolet region (Kahn, 1979).

De Marco *et al.* (1984, 1986) by using ^{1}HNMR examined the change in conformation of met-enkephalin, a pentapeptide which acts at opiate receptors in the brain. They have compared the peptide conformation in water and in dimethyl sulfoxide, in sodium dodecyl sulfate micelles and in AOT reverse micelles. In their hands the opioid molecule undergoes the maximal conformational change in reverse micelles, compared to the other media studied. By contrast, the pancreatic secretory trypsin inhibitor remains highly flexible suggesting no specific interactions with reverse micelles. Salom, Abad and Braco, (1992) have used AOT reverse micelles to discriminate the monomers of gramicidin from the double stranded dimers using CD, since their respective amount varies with the water content of the system (*Figure 11*). By injecting directly micellar samples of the membrane-spanning peptide on a column they were able to monitor the respective monomer-dimer proportions by high performance liquid chromatography (HPLC).

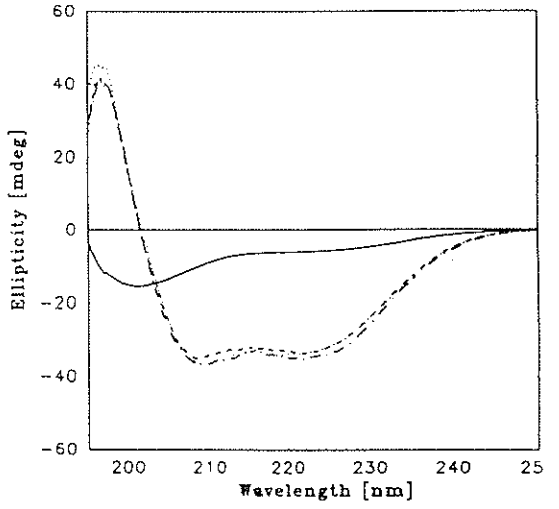

Figure 10. Far-ultraviolet CD spectrum of mellitin in 0.1 M Tris-HCl buffer, pH 7.5 (——), and in reverse micelles of 0.1 M AOT in n-heptane, at Wo=5 (. . . .), Wo=10 (- - - -) and Wo=20 (· — · —) respectively, in the same buffer (Guz and Wasylewski, 1994, with permission.)

REORGANIZATION INTO NON-NATIVE STRUCTURES

A number of investigators have reported structural alterations after protein solubilisation in reverse micelles. These processes can be interpreted from different perspectives and require a careful analysis of the data, before concluding, as many have done, that a denaturation has occurred. Lysozyme and human serum albumin, two well characterized globular proteins, constitute excellent, although quite different examples in this respect.

Lysozyme

The complex behavior of lysozyme in 0.05 M AOT reverse micelles was examined by Grandi, Smith and Luisi, (1981). First, a steady decrease in ellipticity was observed in the far-ultraviolet CD, from −15 000 to −12 000, in a water content range from Wo 3.3 to 33.3 (*Figure 12*). This value was however higher than that obtained in aqueous solutions (−10 000). They suggested that the lysozyme might be in a more rigid conformation. The result corresponds to a decrease in helix from 48% to 42% as compared to the value of 32% helix measured in water (Adler, Greenfield and Fasman, 1973). In addition a large intensity decrease was observed in the near-ultraviolet region as the catalytic activity dropped. The question was subsequently reexamined by Steinmann, Jackle and Luisi, (1986), in the absence and in the presence of inhibitors such as N-acetyglucosamine (NAG) and Tri-NAG (*Figure 13*). After a detailed investigation the authors finally concluded that lysozyme was indeed denatured in reverse micelles of AOT. This did not, however, occur in presence of specific inhibitors or substrates which stabilize the native conformation.

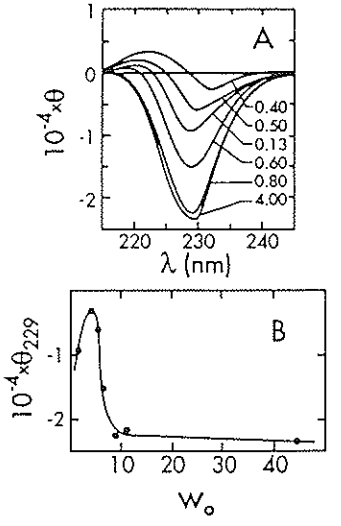

Figure 11. A. Far-ultraviolet CD spectrum of gramicidin in reverse micelles of 0.05 M AOT. The numbers refer to the water percentage in the micellar solutions (v/v). B. Variation of the ellipticity at 229 nm from the curves in A as a function of Wo. (Salom, Abad and Braco, 1992, with permission.)

But then, what is the denaturation mechanism involved? The first suspected was the binding of the anionic surfactant to the basic protein (pHi= 10). If such were the case, the CD spectrum should be affected by the surfactant concentration, but spectra measured at different AOT concentrations were unchanged. However, as is known to investigators working with AOT reverse micelles, the critical micellar concentration of the surfactant in water is low and, in the 0.05–0.1 M concentration range, few AOT monomers are available for protein binding.

Another mechanism has then to be considered. Lysozyme was incorporated into reverse micelles of a neutral (tetraethylene glycol mono ether) or of a cationic cetyltrimethyl ammonium chloride (CTAB) surfactant. It was found that the enzyme retained a high activity in this milieu and that the CD spectrum of lysozyme remained unperturbed, and identical to that recorded in aqueous solvents (Steinmann, Jackle and Luisi, 1986). These results imply the tight binding of the protein to the micellar wall, inducing probably a competition between electrostatic interactions within the protein itself (involving opposite charges of amino acid residues) and the negatively charged sulfonate head groups. Thus, in anionic micelles and in the absence of substrate or inhibitor, the internal stabilizing forces within lysozyme are overcome, leading to the rupture of some intramolecular salt bridges and finally to the perturbation of the overall protein structure.

As a final remark, it is interesting that lysozyme structure comprises two lobes, separated by a cleft containing the active site. The enzyme has a hinge bending mode in which the domains forming the sides of the deep active site cleft, close down or open (Faber and Matthews, 1990). Thus in reverse micelles, any significant opening of the active site cleft of lysozyme would reduce the catalytic activity, increase the space available for side-chain motion and lead to the weaker optical activity, as observed initially in the near-ultraviolet band by Grandi, Smith and Luisi, (1981).

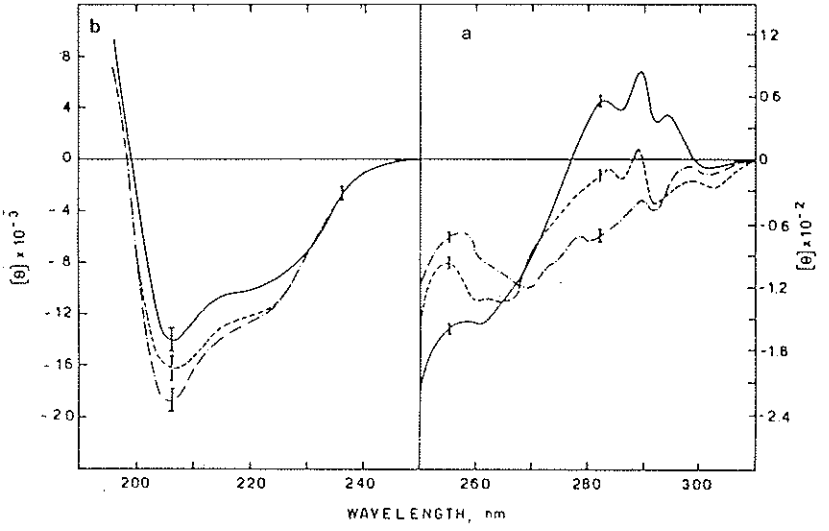

Figure 12. Circular dichroism spectrum of lysozyme in water (——) and in reverse micelles of 0.05 M AOT in isooctane, at 0.3 % (. — . —) and 3% v/v water content (- - - -) a. near-ultraviolet, b. far-ultraviolet. (Grandi, Smith and Luisi, 1981, with permission.)

Human serum albumin

Structural changes of human serum albumin (HSA) upon incorporation into AOT reverse micelles were first reported by Marzola, Pinzino and Veracini (1991a), and

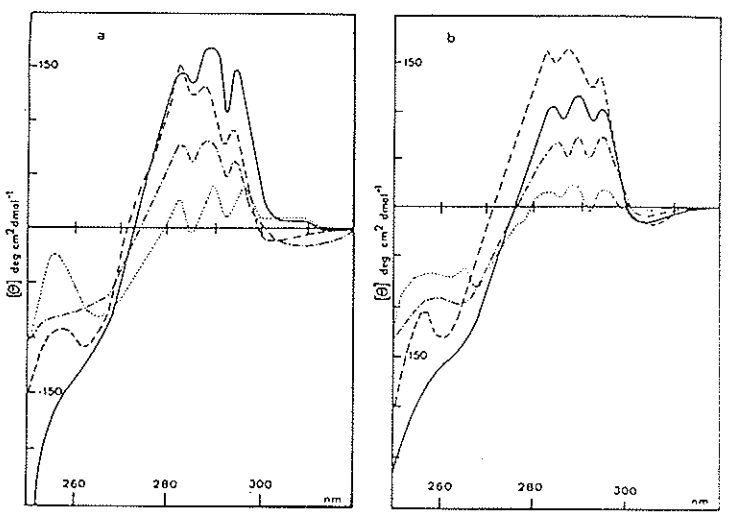

Figure 13. Near-ultraviolet CD spectrum of lysozyme in presence of saccharides. a. Lysozyme-NAG complex in buffer, pH 7.0 (——), and in reverse micelles of 0.05 M AOT in isooctane at Wo values 3.0 (- — —), 6.0 (. — . —.), 9.0 (. . . . .). b. Lysozyme-triNAG complex: in the same buffer (——), at Wo=3.0 (--------), Wo=16 (. — . —) and Wo=25 (. . . . .) respectively. (Steinmann, Jackle and Luisi, 1986, with permission.)

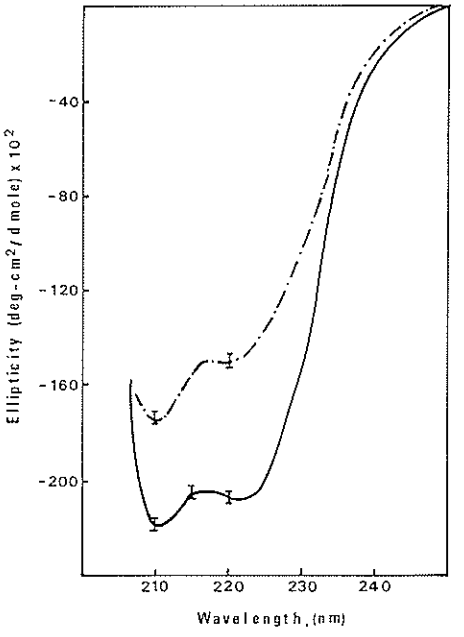

Figure 14. Far-ultraviolet CD spectrum of HSA in 0.05 M Tris-HCl buffer, pH 8.0 (——) and in reverse micelles of 0.1 M AOT in isooctane, at Wo=22.4 with the same buffer (· — · —). Bars indicate experimental error. (Desfosses *et al.*, 1991.)

attributed to a denaturation mechanism. The problem was in fact more complex as shown later by the work of Desfosses *et al.* (1991). These investigators found indeed a loss of intensity in both the near (not shown) and the far-ultraviolet regions of the albumin CD spectrum (*Figure 14*). Secondary structure calculations indicate a loss of approximately 15% alpha helix for HSA at a Wo value of 22.4, in 0.05 M Tris, pH 8.0,. Such an unfolding of a compact, highly structured (68% in alpha helix) macromolecule (He and Carter, 1992) was unexpected. Since aqueous AOT solutions (at subcritical micellar concentration) were unable to unfold HSA, a denaturation mechanism involving the binding of surfactant could be ruled out. Note that these results, however, do not agree with a recent paper of Takeda *et al.*, (1994) who working on bovine serum albumin, report a drop in alpha helix for both micellar and aqueous AOT solutions. The loss of periodic structure was again attributed to the binding of the protein to the surfactant head groups, although in 0.014 M phosphate, at pH 7.0, bovine albumin carries few positive charges and the electrostatic interactions should then be rather weak.

Because HSA is the carrier of a large array of ligands, such as fatty acids, hormones and therapeutic drugs, it was expected that a loss of periodic structure of such amplitude should lead to a decline in the binding properties. To test this hypothesis Desfosses, Nicot and Waks (1992) studied the ligand-binding properties of HSA in AOT reverse micelles. They observed that, at the same time as the protein unfolds, the binding of a fluorescent ligand, dansyl-sarcosine or of a drug, oxyphenylbutazone, was indeed decreased by several orders of magnitude. They attributed the unfolding of HSA in reverse micelles, to a surface-induced conformational change occurring on the

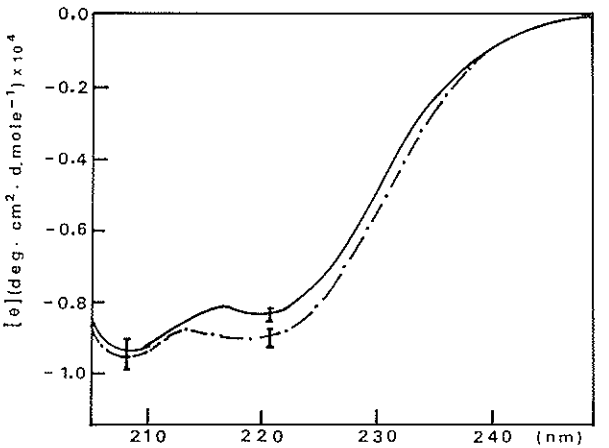

Figure 15. Far-ultraviolet CD spectrum of HSA (49–306) peptic fragment in 0.05 M Tris-HCl buffer, pH 8.0 (——) and in 0.1 M AOT reverse micelles in isooctane in the same buffer, Wo=22.4 (· — · —). Ellipticities are expressed on a mean residue basis. (Desfosses, Nicot and Waks, 1992.)

contact with the micellar wall and different from a denaturation process occurring in bulk solvent.

If this proposal could be substantiated, the unfolding would constitute a ligand-release facilitating mechanism similar to that occurring *in vivo*, upon cellular membrane contact between HSA and the hepatocyte membrane. Such a mechanism was postulated earlier by Reed and Burrington (1989) who suggested that the contact of HSA with the hepatocyte membrane was leading to two subpopulations of molecules. The first would display an enhanced affinity for the membrane and a lowered affinity for its passenger ligands, while in the second the affinities would be reversed. A similar observation had been previously reported by Horie *et al.* (1988) following contact of the protein with the hepatocyte membrane.

To address this issue, Desfosses, Nicot and Waks, (1992) have isolated after proteolysis large fragments of HSA, which retain in buffer and in reverse micelles a substantial amount of periodic structure (*Figure 15*) as well as the unchanged affinity for ligands of the parent molecule. The results demonstrated that in contrast to the native HSA, the (49–306) fragment does not undergo any conformational change in reverse micelles, (indicating that the presence of both the N and C termini are required for the transition), while preserving the full binding capacity for oxyphenylbutazone. They concluded, therefore, that in intact HSA the drug-release facilitating mechanism originates unambiguously from the interaction of the native macromolecule with the micellar interface, inducing a loss of alpha-helix together with the drop of the drug association constant.

A recent paper of Hua *et al.* (1991) is of relevance to the above mechanism. These authors have reported that the interaction of an active insulin mutant (Gly B24) with its receptor involves a partial unfolding of the B-chain with detachment of the A-chain/B-chain contact, thus exposing buried side-chains. The exposure of a hydrophobic amino acid sequence, highly conserved in various species, seems to be required for a direct contact of the hormone with the insulin membrane receptor. In a parallel manner the partial unfolding of HSA alpha-helix

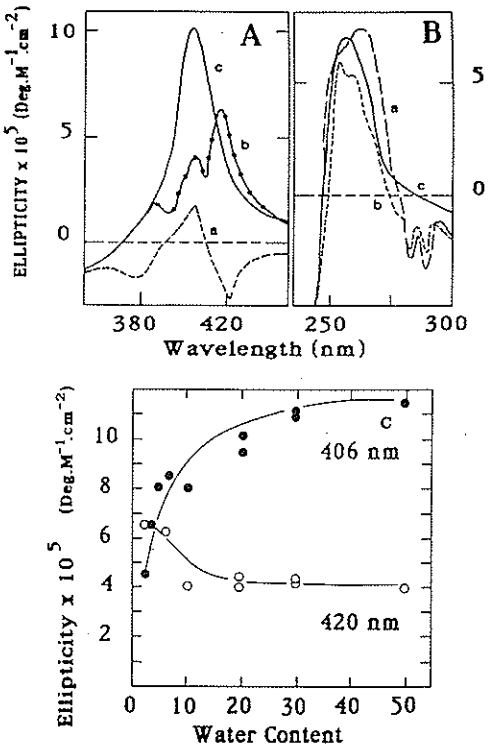

Figure 16. Circular dichroism spectrum of cytochrome C. A. In the Soret band: a. in a neutral pH aqueous solution (— — —), b. in reverse micelles of 0.1 M AOT, at Wo=45 (•-•-•-•), c. at Wo=30 (——). B. In the near-ultraviolet: a. in water (— — —), b. and c. in reverse micelles at Wo values of 10 (----) and 20 (——) respectively. Ellipticities are expressed on a molar basis. C. evolution of the ellipticity of Soret band at 406 and 420 nm with respect to water content of the system. (Brochette, Petit and Pileni, 1988, with permission.)

(or helices) seems to be required for both contact with the micellar interface and for ligand release.

Cytochrome c

A basic (pHi=10.5) protein, cytochrome c (cyt c), constitutes another illustration of an active interaction occurring between a protein and negatively charged micellar interfaces. On the cellular level it has been established that electrostatic interactions play a crucial role in redox processes by modulating both the conformation and the dynamics of the protein, as well as membrane surface (Cheddar and Tollin, 1991).

The interaction of the surfactant sulfonate groups with the positive charges which are segregated in well defined patches on the enzyme surface, determines the location and orientation of the protein in AOT reverse micelles. Brochette, Petit and Pileni, (1988) have shown that the protein is located at the micellar interface, with the heme crevice directed toward it. They have recorded CD spectra of cytochrome c in O.1 M AOT reverse micelles, in the near-ultraviolet and in the Soret region (*Figure 16*). From the changes observed, mainly a large increase in the Soret band intensity, and from a

rearrangement of dichroic bands in the aromatic region, they concluded that the structure of the protein was loosened with increasing water content. These results were interpreted by Hildebrandt and Stockburger (1989) by the presence of a mixture of conformational states I and II identified by resonance Raman spectroscopy. In state I the native conformation is almost conserved, the redox potential of cytochrome c is the same as in water and the low-spin configuration of heme is preserved, while in state II the heme crevice opens and the iron exists in thermal equilibrium between a five-coordinated high-spin and a six-coordinated low-spin.

Vos *et al.* (1987a) have carried out far-ultraviolet CD measurements on cytochrome c in 0.2 M AOT reverse micelles. They report that the ellipticity value at 222nm decreases from −14 100° in phosphate 0.02 M, pH 7.0 to −12 800° in reverse micelles at a Wo value of 5.0, and further down to −6700° at Wo=40. Conversely, the ellipticity of cytochrome c in CTAB-isooctane-hexanol reverse micelles, remains almost unchanged as compared to its value in buffer. This result is a clear indication of the role of electrostatic forces in the destabilization of the protein structure of cytochrome c (since CTAB is a cationic surfactant) and thus presents similarities with the mechanism of lysozyme structural perturbation in reverse micelles.

In conclusion, electrostatic interactions between cytochrome c and the negatively charged sulfosuccinate polar head groups seem to disrupt the forces which maintain the internal cohesion of the protein, leading to a loosening of the tertiary structure. This process might start at a weak structural point such as the axial ligation through Met-80 and lead to the opening of the heme crevice. A similar mechanism has been also suggested during the interaction of the protein with anionic phospholipids (Pinheiro and Watts, 1994). It is suggested that conformational changes may facilitate electron transfer *in vivo* between the mitochondrial enzyme complexes of cytochrome reductase and oxidase, by a better exposure of heme groups.

FUNCTIONALLY IMPORTANT PROTEINS WHICH FIT NO ESTABLISHED SCHEME

Two enzymes have been extensively studied in reverse micelles: liver alcohol dehydrogenase (LADH) and alpha-chymotrypsin (as well as other serine proteases). However in contrast to what could have been expected, conflicting reports concern both their activity and structure. Several parameters can be blamed on the disparity of results and it is difficult to reach firm conclusions from these studies. For example, early authors may not have been aware of impurities included in commercial preparations of the surfactant AOT. In addition to acidic impurities, Luisi *et al.* (1984) have pointed out impurities absorbing between 200nm and 300nm, that could obscure far-ultraviolet spectral measurements of the guest proteins and lead to erroneous results. The commercial origin of enzymes may also interfer with some of the results. Although it might be adventitious, it is amusing that the investigators who have reported comparable LADH conformations in buffer and in reverse micelles have used an enzyme of the same commercial origin.

Liver alcohol dehydrogenase (LADH)

The conformation of LADH in reverse micelles has been studied by CD, fluorescence and phosphorescence spectroscopy. The first measurements were reported by Meier

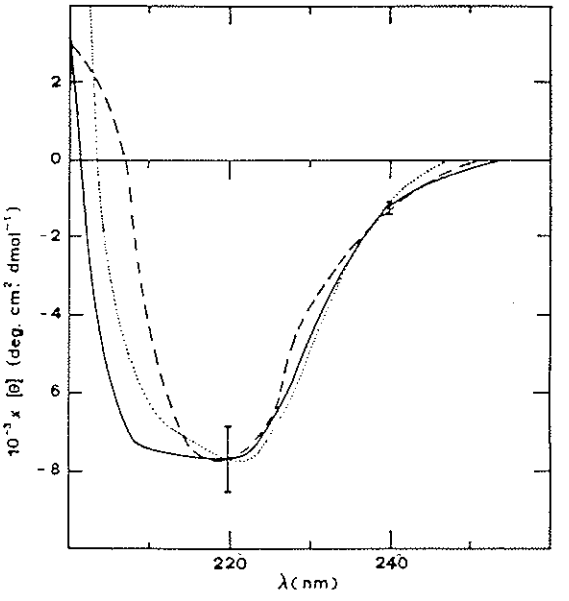

Figure 17. Far-ultraviolet CD spectrum of LADH in 0.1 M phosphate buffer, pH 7.0 (. . . . .) and in reverse micelles of 0.05 M AOT in isooctane. Wo=19 (— —) and Wo=42 (——), in 0.1 M glycine-NaOH, pH 8.8. The instrumental noise is represented by vertical bars. (Meier and Luisi, 1980, with permission.)

and Luisi (1980) who showed, like ribonuclease (Wolf and Luisi, 1979), that the overall native structure of the enzyme was well preserved in reverse micelles, although some minor rearrangements could not be ruled out. As illustrated in *Figure 17,* the spectra of LADH recorded at Wo values of 19 and 42 display minor differences compared to the spectrum recorded in 0.1M phosphate buffer, pH 7.0. Note that in reverse micelles, the enzyme was solubilized in an 0.1 M glycine-NaOH buffer, pH 8.8 with, as a consequence, weaker interactions with the negatively charged AOT polar head groups. In the latter medium the ellipticity minimum at 222nm was around –7500°± 500. The behavior of LADH in reverse micelles has been also investigated by fluorescence (Vos *et al.*, 1987b) and phophorescence as well as by CD spectroscopy (Strambini and Gonelli, 1988). These authors concluded that at Wo=40, where the water pool of an AOT reverse micelle is large enough to accommodate the protein, the overall enzyme conformation is only slightly affected (although CD spectra are not shown).

The question was recently reexamined by Creagh, Prausnitz and Blanch, (1993). These workers have carried out CD measurements in 0.1M phosphate, pH 8.5 and used presumably the same buffer in reverse micelles. In contrast to previous results, they report an increase of ellipticity at 222nm from –4500° to about –7200° upon micellar incorporation, this value remaining constant from Wo=20 to 60. Electron paramagnetic resonance experiments were also carried out: they indicate a significant decrease in rotation frequency when LADH is solubilized in reverse micelles. Furthermore, they confirm only minor changes in conformation when the water content of the system is increased. Finally, from the above results, it appears that investigators seem to disagree more on the ellipticity values of LADH in buffer rather than on its final value after solubilization in reverse micelles.

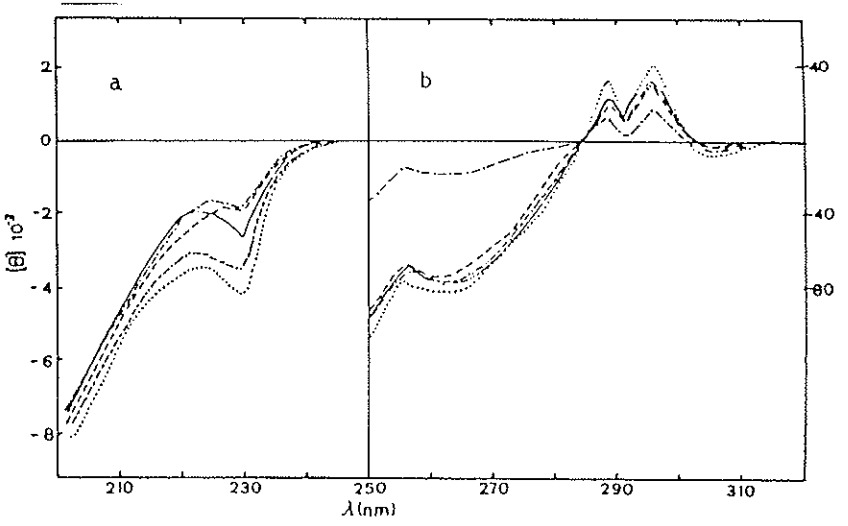

Figure 18. Circular dichroism spectrum of alpha-chymotrypsin. a. far-ultraviolet region in 0.1 M Tris-HCl, pH 7.9 (——) and pH 9.8 (-----). In reverse micelles of 0.05 M AOT in isooctane, Wo=22.5 (· — · —), Wo=18 (— · · —), Wo=13.5 (. . . . .). b. near-ultraviolet region, same symbols. (Barbaric and Luisi, 1981, with permission.)

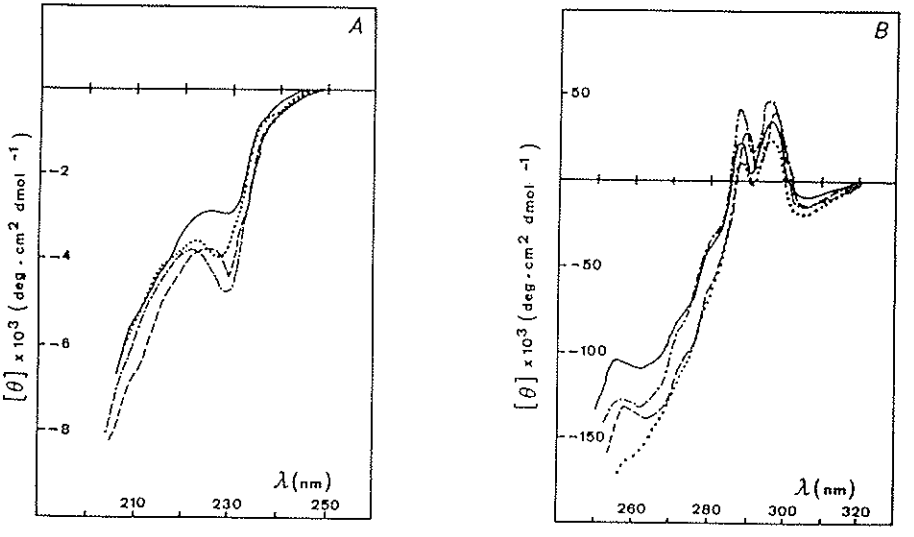

Figure 19. Effect of $CaCl_2$ on the CD spectrum of alpha-chymotrypsin. A. Near-ultraviolet in 0.1 M Tris-HCl, pH 8.5, without Ca^{2+} (——), with 0.05 M Ca^{2+} (-----). In reverse micelles of 0.5 M AOT in isooctane, Wo=10 without Ca^{2+} (. . . . . .), with 0.05 M Ca^{2+} in the water pool (. — . —). B. Far-ultraviolet spectra, same symbols. (Walde *et al.*, 1988, with permission.)

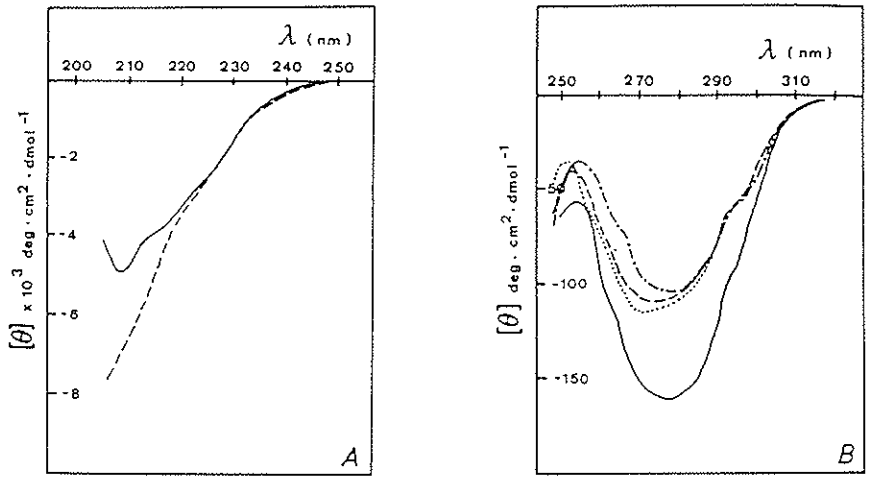

Figure 20. Circular dichroism spectrum of trypsin. A. Far-ultraviolet region. B. Near ultraviolet region; In 0.1 M Tris-HCl buffer, pH 8.5, 0.002 M $CaCl_2$ (——). In reverse micelles of 0.05 M AOT in isooctane, Wo=5.6 (----), Wo=10 (· — · -) and Wo=13.3 (. . . .). (Walde *et al.*, 1988, with permission.)

SERINE PROTEASES

There are a number of conflicting findings about the conformation of alpha-chymotrypsin, the most studied enzyme in reverse micelles. While Menger and Yamada (1979) did not observe any significant modification of the CD spectra of the enzyme, Barbaric and Luisi (1981) in contrast reported substantial spectral changes in the near

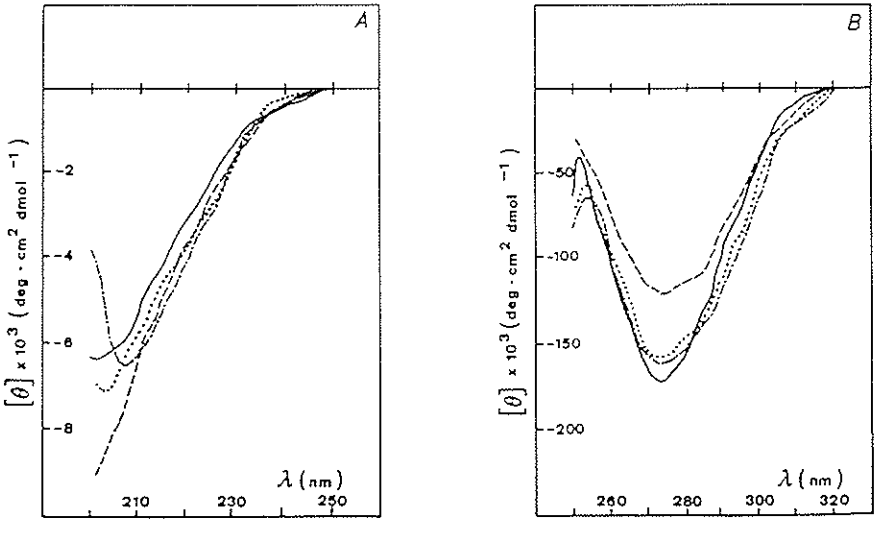

Figure 21. Effect of $CaCl_2$ on CD spectrum of trypsin. A. In the far-ultraviolet region. B. In the near-ultraviolet. In 0.1 M Tris-HCl buffer, pH 8.5 (– – – –). In reverse micelles of 0.05 M AOT Wo=10, without Ca^{2+} or with 0.1 M NaCl in the water pool (-------), with 0.01 M (. . . . . .) or 0.05 M Ca^{2+} (· — · — ·) respectively. (Walde *et al.*, 1988, with permission.)

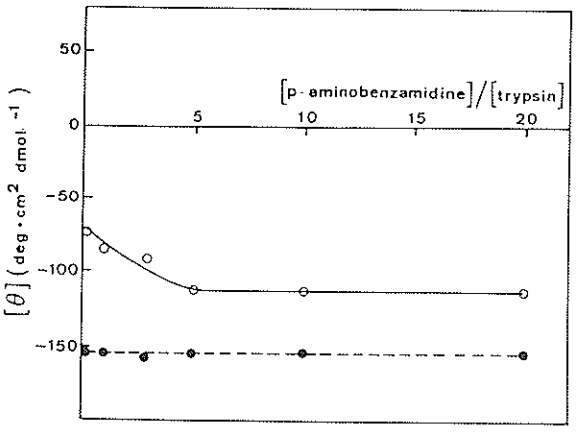

Figure 22. Effect of p-aminobenzamidine on the near-ultraviolet CD spectrum of trypsin. The mean residue ellipticity of trypsin was monitored at 280nm, in 0.1 M Tris-HCl buffer, pH 8.5, 0.02 M in $CaCl^2$ (●●●) and in reverse micelles of 0.05 M AOT, at Wo=10 (OOOO), as a function of the p-aminobenzamidine-to-trypsin molar ratio. (Walde *et al.*, 1988, with permission.)

and far-ultraviolet region, when going from aqueous solutions to reverse micelles, within a Wo range from 13 to 22 (*Figure 18*). These changes were attributed to an increase of the enzyme alpha-helix content. The activity/structure relationship of alpha-chymotrypsin has been reinvestigated in Luisi's laboratory by Walde *et al.*, (1988) and compared to a parent serine protease: trypsin. These authors confirmed their previous findings and, in addition, studied the effect of calcium ions on the CD spectra illustrated in *Figure 19*. After addition of $CaCl_2$ to the Tris-HCl buffer, they observed a further increase of the signal intensity in all regions of the CD spectrum. However, at low calcium concentrations, the enzyme was losing its bound calcium, inducing a partial unfolding of the protein.

The results, however, appear quite different from those obtained with trypsin (*Figure 20* and *Figure 21*). In reverse micelles containing 0.1M Tris buffer, pH 8.5, the ellipticity diminished by 70% in the near ultraviolet while the change was negligible in the far ultraviolet, at 220nm. Here again the addition of 0.05M $CaCl_2$ to the aqueous core of reverse micelles increased moderately the intensity of the spectrum in buffer. Furthermore, the addition of a powerful trypsin competitive inhibitor, p-aminobenzamidine to the enzyme (*Figure 22*) was unable to restore the full native conformation. Walde concluded that in trypsin the lower intensity of the near utraviolet CD spectrum observed in reverse micelles was probably the result of two different mechanisms: a loss of calcium ions from the enzyme binding site and a conformational change in the absence of an excess of inhibitor/ substrate concentration.

An attempt at a more precise estimation of the alpha-chymotrypsin secondary structure changes in reverse micelles has been carried out by Qinglong, Huizhon and Jiayong (1994). Using FTIR spectroscopy they estimated the loss of alpha-helix to be 7%, and that of beta-sheet about 12%. At the same time an increase of random conformation up to 39% was observed, the beta-turns increasing also by 12%. These

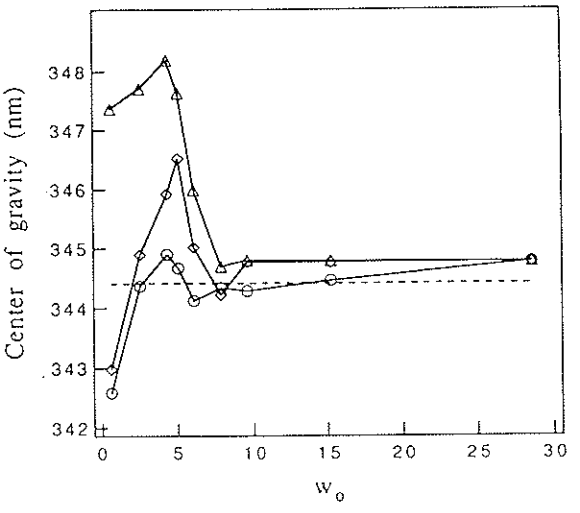

Figure 23. Fluorescence spectrum of alpha-chymotrypsin. The center of gravity of the tryptophan fluorescence in 0.1 M AOT reverse micelles, in n-octane is represented as a function of Wo. Without incubation of the enzyme (OOOO); after 10 minutes of incubation (◊◊◊) at a constant initial Wo=28.6 value; after 10 minutes of incubation at Wo values shown on the abscissa (ΔΔΔ). The catalytic activity of the enzyme in 0.10 M Tris-HCl buffer, pH 8.2, is indicated by a dashed line. (Dorovska-Taran, Veeger and Visser, 1993b, with permission.)

measurements carried out in D_2O, not in buffer and, in the absence of calcium ions or inhibitors, and thus render comparisons with previous data difficult. Finally, a recent paper by Creagh, Prausnitz and Blanch (1993), at variance with the already mentioned literature, reports little change in the secondary structure of the enzyme, except at the lowest water content (around Wo=5.0), where the protein structure is constrained.

Other investigators have tried different technical approaches to the problem, to shed light on the relationship between activity and conformation in chymotrypsin. Direct labelling of the enzyme active site with spin labels (Marzola *et al.*, 1991b; Creagh, Prausnitz and Blanch, 1993; Kommareddi, O'Connor and John, 1994) led these authors to the conclusion that the active site dynamics were essentially unperturbed, rotational restrictions being seen only at low water content (Wo< 10). Several interesting investigations concerning the interaction of the enzymatic site with water molecules have been published by Dorovska-Taran, Veeger and Visser (1993a). By labelling the active site of alpha-chymotrypsin with an anthraniloyl fluorescent label, the authors, using time-resolved fluorescence, have observed that at low water content the enzyme is predominantly present as a single conformer characterized by a long fluorescence lifetime and a high fluorescence quantum yield. By increasing the water content of the system, a broad lifetime distribution appears which is very close to the conformational pattern of the enzyme in buffer.

In a subsequent paper Dorovska-Taran, Veeger and Visser (1993b) have studied the involvement of different water structures in the development of the catalytically active conformation of chymotrypsin. For example, they remarked that at the very low Wo value of 0.65, the protein is inactive as if 'frozen': this result confirms that if it is necessary, enzymatic activity requires a certain amount of flexibility. In fact, at a higher Wo range from 0.65 to 2.3, the protein becomes even more rigid until Wo=5,

where the enzyme displays a minimum of activity. The latter water content corresponds to the maximum red shift of the tryptophan fluorescence center of gravity, indicating that the average of the polarity around the enzyme molecule is even higher than in water (*Figure 23*), a result difficult to understand. A further relatively small water increase (to Wo=8.0) provokes a burst in enzyme activity. This effect is attributed to a cooperative binding of water molecules to the protein, leading to the appropriate hydration of the enzyme. Water thus affects the reaction rate both by hydration of the protein and as a reactant. In a similar manner Walde *et al.*, (1988) found the optimum activity of trypsin at a Wo value around 10, where the amount of water present is only sufficient for a few layers around the protein molecule. In this relation it is interesting that Fletcher, Robinson and Tabony (1988), using quasi-elastic neutron scattering, observed that only 450 water molecules out of a total of 4800 were tightly associated with one alpha-chymotrypsin molecule at Wo=20, corresponding to an approximately half-completed monolayer coverage of the enzyme.

Other lipases

Human pancreatic lipase has been investigated in reverse micelles by Walde, Han and Luisi (1993). Circular dichroism spectra reveal, as in many other proteins, only minor changes in the far-ultraviolet spectrum in response to the aqueous environment (*Figure 24A*). Several changes in the near-ultraviolet bands of the spectrum (*Figure 24B*) where aromatic residues are predominant, are in good agreement with the alteration of the intrinsic fluorescence properties of the lipase. According to the authors, CD and fluorescence measurements can be interpreted by a change in the microenvironment of at least one of the seven tryptophans or one of the 15 tyrosines. Although the conformation of the polypeptide backbone remained almost unper-

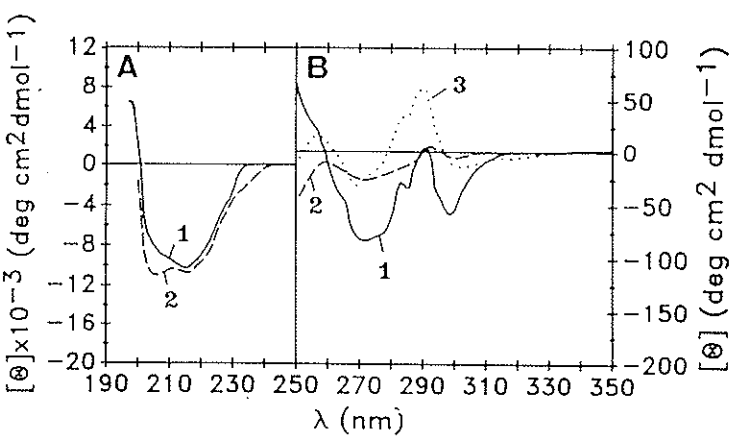

Figure 24. A. Far and B. near-ultraviolet CD spectrum of human pancreatic lipase. 1. In 0.05 M Tris-HCl buffer, pH 7.5. 2. In reverse micelles of 0.05 M AOT, in isooctane, Wo=10 with the same buffer. 3. as in 2. in the presence of 1×10^{-5} M tetrahydrolipstatin. (Walde, Han and Luisi, 1993, with permission.)

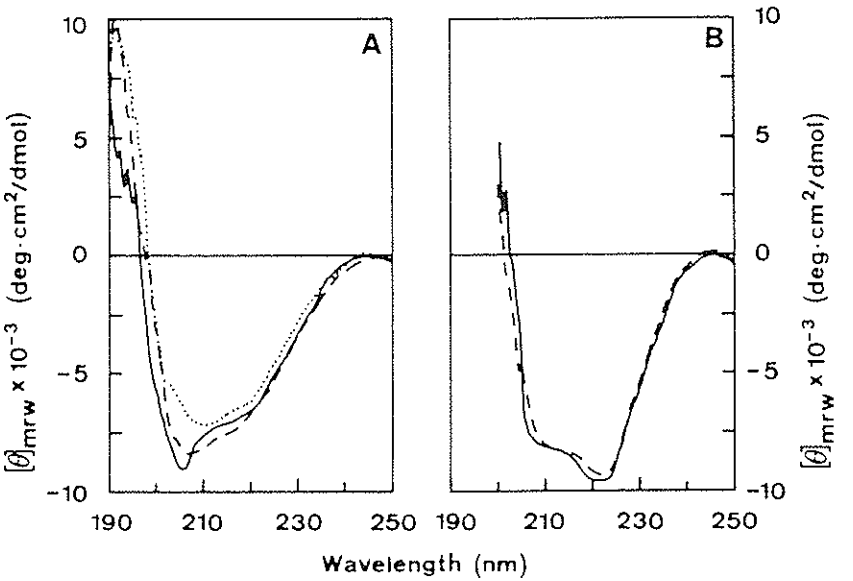

Figure 25. Circular dichroism of *Candida rugosa* lipase in reverse micelles of 0.05 M AOT, in isooctane. Effect of the water content on the far-ultraviolet spectrum. A. Wo is caried by adding 0.075 M Tris-HCl buffer, pH 8.1: Wo=10 (. . . .), Wo=20 (------), Wo=30 (——). B. in presence of 0.01 M oleic acid: Wo=8 (— — —) and Wo=20 (——). (Walde, Han and Luisi, 1993, with permission.)

turbed, the authors have observed that in the absence of substrate, the activity of the enzyme is lost in half an hour. When the enzyme was premixed with human or porcine colipase at a molar ratio around 4.0, the activity was maintained, indicating a stabilizing role for colipase.

In fact, these results cannot be explained on the basis of the X-ray crystallographic data for human lipase-procolipase complex (van Tilbeurgh *et al.*, 1993), showing colipase at a distance from the enzyme active site. In order to test whether the binding of inhibitors might stabilize the enzyme conformation, Walde, Han and Luisi (1993) have added tetrahydrolipstatin, a selective covalent inhibitor which binds as an ester to the residue (ser-152) of the active site to pancreatic lipase. Even though the enzyme inhibition and the modified CD spectrum were stabilized for a long period of time, changes were observed in the aromatic region of the spectrum indicating that the native conformation remains perturbed (*Figure 24B*).

Candida rugosa lipase exhibits another example of a rather complex enzyme behavior in reverse micelles. Upon incorporation, a shift of the ellipticity minimum is observed (from 210 to 205nm) attributed to a loss of alpha-helix, in particular at high Wo values (*Figure 25*). However as illustrated in the same figure, the presence of oleic acid restores the conformational stability of the enzyme to its initial value measured in aqueous solvents (Walde, Han and Luisi, 1993).

It can be concluded that in contrast to the predictions of Marangoni (1993), a general pattern of structural modifications, catalytic activity and the relationship between these parameters for all lipases in reverse micelles does not seem to emerge from the data, despite an abundant literature. By contrast, it seems that reverse micelles

emphasize the specific properties of different lipases and their stabilization in presence of substrates or inhibitors.

Protein-protein interactions

Because of their central importance in a number of biological processes (protease-inhibitor complexes, antibody-antigen reactions, self-association of subunit enzymes), the occurrence of protein-protein interactions in reverse micelles has been addressed by several authors

TRYPSIN-INHIBITOR

A typical protein-protein interaction is represented by the binding of trypsin with its inhibitor. From kinetic inhibition experiments, Walde *et al.*, (1988) concluded that exchange and recognition of macromolecules (trypsin and inhibitor) could take place in micellar solutions at the same rate as with low molecular weight molecules. This view has been challenged by Bru and Garcia-Carmona (1991) who found the exchange rate constant to be a thousand times slower when the macromolecules interacted with each other rather than with a small molecule. However, these results provided only indirect evidence of protein-protein interactions in reverse micelles.

MYELIN BASIC PROTEIN PROTEOLYSIS

Although proteolytic enzymes have been widely investigated in reverse micelles their catalytic activity was in general measured using small synthetic substrates. At the cellular level, the substrates of proteolytic enzymes are more usually proteins. Thus to establish whether proteolytic enzymes were able to interact with macromolecules in reverse micelles, a more direct approach could consist in isolating the products of the protein-protein reaction rather than following inhibition kinetics. Nicot *et al.* (1993) have directed their experiments toward this goal. They investigated the digestion of myelin basic protein in reverse micelles by three proteolytic enzymes, trypsin, cathepsin D and *Staphylococcus aureus* V8 protease. The resulting peptides were isolated by HPLC, analyzed by gel electrophoresis and sequenced. It was found that in contrast to proteolysis in aqueous solutions, the number of cleavage sites was considerably decreased with micellar digestion generating long hydrophobic peptides, thus reflecting the large change in conformation undergone by the protein. The cleavage sites obtained in reverse micelles delineate a protein hydrophobic domain protected from proteolysis, with a potentiel beta-sheet conformation (Stoner, 1984) interacting with the native myelin membrane.

ANTIGEN-ANTIBODY INTERACTION

Another approach for the experimental study of specific protein-protein interactions in reverse micelles was carried out by the direct measurement of antigen-antibody binding, using fluorescence polarization spectroscopy. Groome *et al.* (1990) have obtained a Fab fragment from a monoclonal antibody raised against the aqueous form of MBP. It was titrated in buffer with the dansylated protein itself and with a

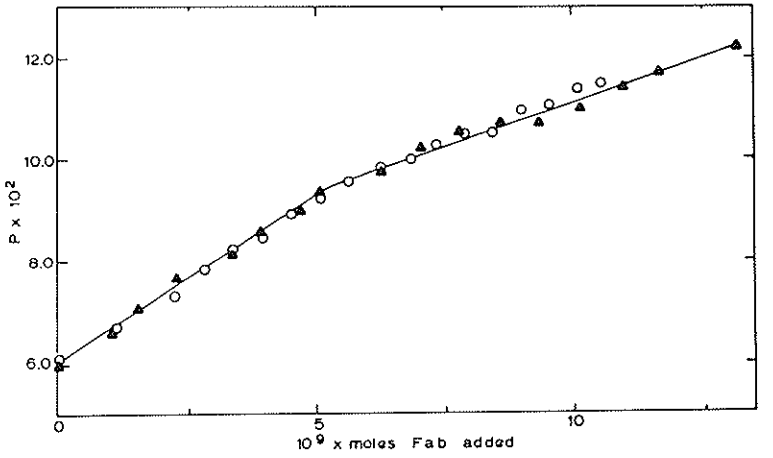

Figure 26. Dependence of fluorescence polarization (P) in arbitrary units, on the formation of the MBP dansylpeptide-Fab complex. The titration curve was carried out in reverse micelles of 0.1 M AOT, in isooctane, at Wo=22.4 with 0.05 M Mops (4-morpholinopropane sulfonate) buffer, pH 7.15. Different symbols represent different monoclonal antibody clones. The combining ratio was one gly(119)-gly(131) peptide molecule per Fab fragment. (Groome *et al.*, 1990.)

dansylated synthetic human MBP peptide gly(119)-gly(131). The monoclonal antibody responded to both the dansylated protein and peptide. In reverse micelles, analysis of the fluorescence polarization titration curve with the peptide reveals a specific protein-peptide interaction with a molar stoichiometry of 1:1 (*Figure 26*). In addition, the Fab fragments have retained the high affinity for the peptide of the same order of magnitude as that for intact MBP, both measured in buffer (with an association constant of 4.10^8 M^{-1}). However in contrast to aqueous solutions, no binding of Fab was observed with the intact protein itself in micelles. The latter result was expected as a consequence of the large refolding of MBP observed when going from water to reverse micelles (Nicot *et al.*, 1985). Antigen-antibody reactions can thus also serve to probe a protein conformational alteration after micellar incorporation.

DISSOCIATION OF SUBUNIT ENZYMES

The degree of dissociation induced by reverse micelles in multisubunit enzymes belongs to any account of protein-protein interactions and constitutes yet another topic of discussion and controversy (for a critical discussion see Oldfield, 1994). A number of reports have addressed this issue, but few of them have involved direct physical measurements of the phenomenon. Russian authors, in particular, have relied almost exclusively on the so called 'bell shaped curve' of enzymatic activity plotted as a function of the calculated Wo value, as a measure of the dissociation of enzymes into active subunits (Kabanov, Nametkin and Levashov, 1990; Kabanov *et al.*, 1991a,b). By a somewhat different approach, Kabakov, Klyachko and Levashov, (1994) have dissociated the alpha and beta subunits of penicillin acylase by gel filtration under denaturing conditions. After removal of urea by dialysis, the subunit activity was tested in buffer and in AOT reverse micelles: in both cases a very modest value for the catalytic activity was recorded.

According to these authors, a good correlation was nevertheless found between the optimal activity recovered from separated enzyme polypeptide chains and the calculated Wo value, corresponding to the size of the smallest micelle capable of hosting a single subunit. In this regard it is interesting to keep in mind that, according to Lindsay and Pain (1990), penicillin acylase, a spherical, stable enzyme, is resistant to denaturants. In buffer, separation of its constituent alpha (catalytic) and beta (binding) subunits leads to unfolding and loss of activity. It still remains to be confirmed whether penicillin acylase behaves in a different manner in AOT reverse micelles. Note that contrary to Rahaman and Hatton (1991), the approach of the Russian authors precludes any redistribution of surfactant and water between the protein-free and the protein-filled micelles. However, no consensus solution to this problem has been reported so far (Levashov *et al.*, 1982; Chatenay *et al.*, 1985; Zampieri, Jackle and Luisi, 1986) and the issue remains unresolved.

Khmelnitsky *et al.*, (1993) on the other hand, studied the dissociation of the tetrameric lactate dehydrogenase in buffer and in AOT reverse micelles by time-resolved polarized fluorescence spectroscopy of tryptophan residues. When the size of the enzyme considerably exceeded the initial size of the micellar aqueous core, the protein was forced to dissociate into smaller species. This finding implies that during the solubilization process the protein creates its own tailor-made micelle around itself, as suggested previously by Chatenay *et al.* (1985) for MBP. The final micellar size originates therefore from the competition between forces of solubilization and forces of tetramer dissociation. It has to be underlined that in the latter work, the fluorescence technique does not allow discrimination between enzyme monomers and dimers.

Another case of subunit dissociation in AOT reverse micelles has been described by Chang, Huang and Chon, (1994), studying the tetrameric pigeon-liver cytosolic malic enzyme. To confirm the splitting of the enzyme in reverse micelles of AOT, the investigators chemically cross-linked the subunits at various values of Wo, i.e. at various micellar sizes. From activity measurements, and also from gel electrophoresis of the crosslinked subunits, they concluded that the tetramer-to-dimer dissociation constant of the oligomeric enzyme was increased by one or two orders of magnitude in the micellar solution. In addition, they observed that both monomers and dimers had the same specific activity in reverse micelles, but unfortunately the catalytic activity was only 10% of the initial value measured in aqueous solution. The authors suggest that the inactivation process may originate from the interaction between the subunits and the inner micellar wall, without yielding any experimental evidence. As will be discussed below, other types of inactivation mechanisms, for example a close interaction between catalytic sites and interface subunit integrity, may also play a significant role in connection with oligomeric enzyme denaturation.

REFOLDING AND ASSOCIATION OF SUBUNITS

An interesting example of enzyme association-dissociation mechanism in reverse micelles has been reported by Garza-Ramos *et al.* (1992) and concerns a homodimeric enzyme: triosephosphate isomerase (TPI). After denaturation in guanidine hydrochloride and splitting of the enzyme into monomers, the authors succeeded in refolding and reassembling the subunits, provided the amount of water present in the micellar solution was high enough (6%). The experiments were carried out with micelles of

cetylmethylammonium bromide (CTAB), a cationic surfactant, hexanol as a cosurfactant and n-octane. In another experiment, TPI was covalently derivatized at a residue of the catalytic site (glu-165) with 3-chloroacetol phosphate (CAP), thus allowing the formation of heterodimers possessing a single active site. From kinetic experiments the authors observed that in reverse micelles the reactivation time of the denatured enzyme monomers dropped at higher protein concentration. They concluded, therefore, that the limitation of the overall sequence of TPI reactivation in reverse micelles was controlled by formation of dimers remaining highly stable in the micellar solution (only 10% of activity loss in five days). A notable result is that, once again, TPI active monomers could not be detected. These results point clearly to essential water requirement for a correct refolding and reassociation during the formation of oligomeric enzymes. The existence of a stable catalytic heterodimer with a single active site demonstrates the close interaction between the remaining active site and the maintenance of the subunit interface integrity (Sun, Yuksel and Gracy, 1992).

The problem was investigated further by Fernandez-Velasco *et al.*, (1995). Using fluorescence emission spectroscopy, the authors were able to distinguish between the refolded monomers which are either competent or unable to assemble into active dimers. The results of this work have been discussed in the paragraph dealing with the refolding of proteins (*vide supra).* In summary it can be said that in CTAB reverse micelles, both relatively high water concentrations (5%to 6%) and internal structural rearrangements at the interface between two monomers are prerequisites for catalytically active TPI.

In this context it is interesting to recall the results of Sun, Yuksel and Gracy, (1992) concerning the refolding and dimerization of TPI in buffer. These authors have demonstrated that the original enzyme structure is never fully recovered in refolded active dimers. One wonders whether this finding does not apply also to micellar solutions of TPI. Unfortunately the work of Fernandez-Velasco *et al.* (1995), lacking essential structural information, does not result in a clear answer to this issue. Recent information on the molecular basis of enzyme activity, may render the situation still more intricate. It has been shown by Xue and Yeung (1995) that individual enzyme molecules (which can be hosted as such in reverse micelles) may display variable activities by stable conformational arrangements of their subunits, making certain active sites less accessible than others for reaction. This is yet another example where it would be adventurous to infer a native enzyme conformation solely from the kinetics of catalytic activity in reverse micelles.

Recent and innovative applications

A number of applied aspects of reversed micelles including a large array of biotransformations and protein separation techniques has been documented by Oldfield (1994). We will not duplicate this description. Rather we have selected several recent applications which present either unusual biological interest or an original approach for protein separation. Even though the system displays many desirable features for large scale processing, micellar separation depends on numerous parameters which have to be redefined for each class of proteins or protein mixtures, and a careful choice of extraction conditions (Kadam, 1986, Marcozzi *et al.*, 1991). The contribution of multiple interactions between polar head groups, hydrophobic tails, organic and water

phases and so forth, in addition to the rich polymorphism of amphphilic phases renders the behavior of the separation system complex and difficult to predict.

NONIONIC SURFACTANTS

Strong electrostatic interactions are often held to be responsible for protein destabilization (Honig and Nicholls, 1995), leading to structural disruption, as described above for cytochrome c and lysozyme in AOT reverse micelles, and as a consequence to impaired catalytic properties. This difficulty can be circumvented by the utilisation of nonionic surfactants. One of the first cases of protein extraction by reverse micelles of a biodegradable, nonionic surfactant has been reported by Ayala *et al.* (1992) and the system was thereafter explored in detail by Komives, Osborne and Russell, (1994) from the same laboratory. Reverse micelles made of a polyoxoethylene sorbitan trioleate (Tween 85) in hexane, with isopropanol as cosurfactant, were successfully used to extract cytochrome c, hemoglobin (in which the heme group is not covalently bound to the protein) and a proteolytic enzyme, subtilisin. It appears that for each set of proteins experimental parameters must be optimized as shown by Komives, Lilley and Russell, (1994) for an organophosphorus hydrolase active in pesticide degradation. These authors report a detailed analysis of the relationship between micellar structure and enzyme activity. In particular, the importance of the cosurfactant in the partitioning of the enzyme between the surfactant and the water pool is established. It is shown to be related to the variation of the interfacial tension at the surfactant layer, resulting from the presence of the isopropanol in the system.

PROTEIN ENGINEERING

Since electrostatic interactions constitute the major driving force for solubilization (Nicot *et al.*, 1985) and/or transfer between hydrophilic proteins and AOT reverse micelles (Leser *et al.*, 1986; Hatton, 1989), point mutations can be performed to increase the electrostatic potential of macromolecules. Pires *et al.* (1994) have modelled the structures of native and mutant rat cytochromes b_5. After calculating their respective electrostatic potentials, they have predicted an eventual improvement in the extraction process. As expected, the replacement of Glu by Lys residues resulted at the same time in an increase of the positive surface potential of the enzyme, in a stronger interaction with the negatively charged micellar interface and in a more favourable extraction efficiency. In contrast, a complete correlation between protein dipole moments and extraction yields could not be established. There are however at least two prerequisites for a successful operation: (i) a model of the protein structure has to be available for the search for the most efficient substitutions, and (ii) this type of approach will work only for proteins whose genes have been cloned, which can be altered and expressed. Obviously, it is also assumed that the mutations do not affect the native folding of the protein. This work constitutes a good example of rational engineering of protein surfaces with a view to operational protein extraction.

AFFINITY PARTITIONING

Among the many protein extraction systems using reverse micelles, those based on

affinity partitioning deserve comment. In early experiments, Woll *et al.* (1989) have utilized octyl glucoside to extract concanavalin A, while Coughlin and Bacłaski (1990) purified avidin from an aqueous solution using an alkylated derivative of biotin. By a somewhat different approach, Paradkar and Dordick (1993) have proposed an affinity-based reverse micellar extraction and separation of proteins (ARMES). Their strategy integrates affinity interaction with the selective extraction of an affinity complex. The complex is extracted by phase separation from water by a reverse micellar solution at pH 5.0, in order to remove competing proteins. The interesting point is that selectivity of the separation process is obtained in both the affinity and in a second extractive step in the presence of concanavalin A. This enables the selection of lower binding affinities for ligand-ligate complexes than that required with affinity chromatography. With concanavalin A as a ligand and peroxidase (SBP) from crude soybean hulls, 30% of the initial SBP activity has been extracted. As often happens, difficulties occurred in the back extraction step of the enzyme into the aqueous phase. They were simply solved by raising the temperature up to 40°C. A purification factor of thirtyfold was achieved for SBP, paralleled by a complete regeneration of concanavalin A, which allowed a second ARMES cycle resulting in an overall yield of 58%. During the complete process the enzyme remained active. The authors emphasize the simplicity and the good performance of ARMES as compared to more traditional techniques. Furthermore, the ligand can be regenerated with a 95% efficiency. An extension of the technique to the use of Fab antibody fragments as ligands is anticipated which might dramatically increase the range of ligates to be purified in the future.

Kelley, Wang and Hatton (1993a, b) have undertaken a systematic study of affinity systems by the use of biospecific cosurfactants, consisting of hydrophilic ligands derivatized with hydrophobic tails. The ligands are recognized and specifically bound by proteins. It is assumed that the ligands do not disturb the micellar structure, and interact selectively and reversibly with the protein to be extracted. In their work, the authors have utilized small concentrations of three different types of ligand: alkyl glucosides, boronic acids and phospholipids. They show that the protein association mechanism with its ligand is a competitive process between high and low affinity ligands. This allows extension of both the operating range of pH and salt concentrations over which the protein can be extracted.

Furthermore it was also shown that an optimal cosurfactant tail length is required for the maximum protein intake. Thus, for any given association constant an optimum tail length has to be selected, in particular for proteins displaying rather small ligand dissociation constants. The extraction process is accounted for in terms of difference in interfacial activity of both the free cosurfactant and the protein-cosurfactant complex. Unfortunately the investigators do not proceed to the back extraction of proteins into the aqueous phase. This step often presents many difficulties in terms of surfactant binding to protein, recovery yield and often denaturation at the water-organic solvent interface. Although the system has been modelled in order to predict optimal extraction yields, as yet no large scale industrial processing by this technique has so far been reported.

ISOLATION OF ISOENZYMES

A number of enzymes consisting of isoenzyme mixtures of various activities, display

a wide range of isoelectric points. The recovery of the highest catalytically active isomers is always difficult to achieve. Horseradish peroxidase (HRP) belongs to this family of enzymes and is widely used in enzyme immunoassays, requiring thus a highly purified form of the enzyme. Regalado, Asenjo and Pyle (1994) have carried out purification studies of HRP using AOT reverse micelles. A detailed investigation of extraction conditions showed that the system could achieve high peroxidase recovery by using proper pH, ionic strength, temperature and phase ratio. A curious aspect of the purification process is that the recovery of activity yield increased up to 136%. Isoelectric gel electrophoresis titration curves revealed the origin of the phenomenon: a reduction in the isoenzyme number with preferential concentration of the most active one. Micellar solubilization and extraction have thus achieved the purification of the commercial protein and the enrichment in the isoenzyme of the highest isoelectric point and activity. These results may explain, in part, the much sought after superactivity phenomenon reported by Martinek *et al.* (1986) for the same enzyme.

PURIFICATION OF IMMUNOGLOBULIN FAB FRAGMENTS

In recent years there have been many applications in clinical diagnostics, medical imaging, and targeted drug delivery resulting from the recovery of large amounts of homogeneous monoclonal immunoglobulins of the IgG class, produced by hybridoma cells. A large scale processing of immunoglubulins in reverse micelles is therefore in demand and has been proposed by Kamihira *et al.* (1994). The fractionation scheme is presented in *Figure 27.*

The process involves:

(1) The addition of solid-phase papain, a specific proteolytic enzyme, to the immunoglobulins. After digestion, the remaining solid papain is removed by centrifugation or membrane separation.

(2) The solution containing the smaller Fab and Fc fragments, both prepared to minimize non-specific binding by the intact immunoglobulin, is then incorporated into a micellar phase around pH 8.0, and undigested IgG is trapped in the micellar solution while both Fab and Fc remain in the aqueous phase.

(3) Another micellar extraction separates the two fragments: Fab is extracted in the micellar phase at pH 7.0 while Fc remains in the aqueous phase.

(4) Finally Fab seems to be back-extracted without substantial problems.

A significant point in the processing system which is worth noting, is that back extraction requires the Fab fragment to be first extracted and solubilized by a solution of 6M guanidine hydrochloride, after addition of a micellar disrupting agent, ethyl acetate. The sample is then 20 fold diluted with phosphate buffered saline solution, pH 7.4 and dialyzed in the same buffer. If the dilution of the guanidine solution is less than 10 fold, the authors failed to obtain an active fragment possibly due to the formation of inactive and/or unfolded protein (or fragment) self-association. It is therefore essential to select experimental conditions which minimize the formation of aggregates during the refolding process. Nevertheless, the lack of precise information about the fragment conformation precludes a better understanding of the renaturation mechanism described.

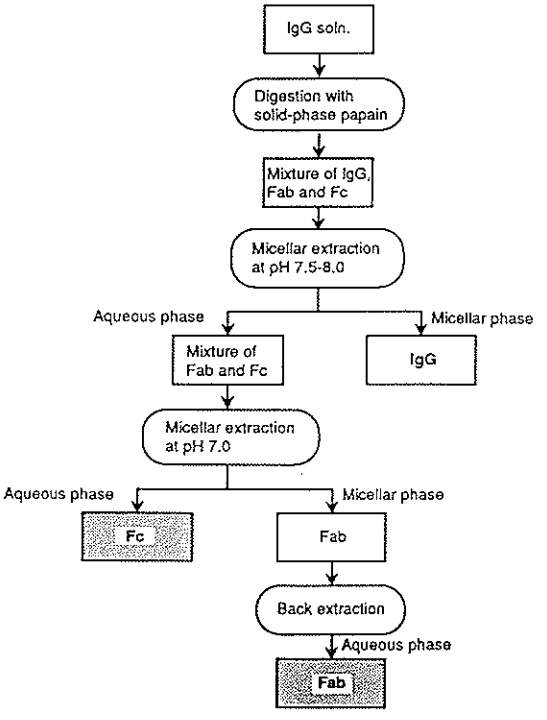

Figure 27. Separation scheme for the fractionnation of Fab and Fc immunoglobulin fragments using reverse micellar extraction (Kamihira *et al.*, 1994, with permission.)

Under these conditions the authors claim that all the extracted Fab is recovered in an active form. In their work, the starting material consisted in polyclonal immunoglobulins which are heterogeneous in nature and display irregular isoelectric point values. The authors suggest therefore that the use of monoclonal antibodies should achieve a satisfactory separation of Fab fragments. The investigators assert that mass separation of antibody fragments is now at hand.

Conclusion

The ordered, periodic, structure and the reactivity of proteins can be significantly altered by their incorporation into the aqueous core of reverse micelles. The observed modifications do not follow well-established general patterns, but rather reflect the great diversity of macromolecular structures and their intrinsic physico-chemical properties. A protein has indeed to adapt itself to a new microenvironment that is anisotropic and more complex by comparison with conventional solvents. It has been suggested that in diluted aqueous solvents, a macromolecule has to comply with at least two opposing requirements: to minimize its free energy and to optimize its biological function (Shoichet *et al.*, 1995). For example an enzyme has to recognize a transition state for substrate transformation or processing, a protein has to identify another protein, a ligand has to bind a transport protein until its final target destination.

It should threfore be possible, by taking advantage of the wide range of forces existing in reverse micelles, to alter or shift the resulting balance in different directions.

Yet, in a broader context, the role of weak forces and their contribution to the overall energetics in this milieu will have to be taken into account. For example, as mentioned earlier, they can induce perturbations in both the conformation and reactivity of confined molecules. 'As the size of a confined macromolecule (or macromolecular aggregate) approaches the size of the confining volume element, considerations of volume exclusion dictate that the configurational entropy of the confined particle will be significantly reduced and its chemical potential correspondingly increased relative to unbound or bulk phase' (Minton, 1995). Inside a tailored-to-measure micelle or when the radius of the aqueous core is reaching the size of the hydrodynamic radius of the encapsulated protein, we are clearly in a situation resembling that described above.

Such forces which can be modulated in reverse micelles contribute presumably to the stability of proteins, stability which is further enhanced by a restricted macromolecular freedom. In this connection, the thermodynamic stability of several globular proteins has been examined in reverse micelles by Battistel, Luisi and Rialdi, (1988) using differential scanning calorimetry. It appears that the maximal stability corresponds to the lowest amount of water present when almost all water is hydration water, *i.e.* only sufficient to form one or two layers around the protein molecule. This is the case when the inner micellar radius is inferior in size to that of the protein, at Wo values under 5.0, when a probable redistribution of water and surfactant between the protein-depleted and the protein-containing micelles occurs. Under such conditions the macromolecular energy is shifted to lower values of the energy scale and denaturation in the unfolding sense probably requires much greater energy input than is observed in water.

What lessons can we learn from studies of proteins in reverse micelles and what are the new directions to look at? First, reverse micelles should be considered as versatile tools for a better understanding of the interactions that govern the various equilibria in macromolecules, and as yet the tool is insufficiently used. Reverse micelles create an internal protected environment of experimentally defined size that harbors functional sites and can protect them. In this context, they bear a resemblance to chaperones with regard to the assistance they can bring in the fine tuning of protein or peptide folding and by preventing aggregation reactions, although the comparison should not be taken too far.

Second, reverse micelles are considered as a highly flexible microreactor, the applications of which remain still largely unexplored (Pileni, 1993). Nevertheless, new applications will require a detailed examination of the protein behavior within the system as well as a careful investigation of the possible macromolecular effects induced on the architecture of the organized bioassembly itself (Chatenay *et al.*, 1987). It is known that such systems display very different structural arrangements, depending on the concentration of their components and are very sensitive to chemical and physical conditions (Komives, Osborne and Russell, 1994).

Third, from a wider perspective, interfacial phenomena and surface-protein interactions play a key role in the organization and the control of biological membranes. The very large interfacial surface generated by reverse micellar solutions is expected to amplify any of these effects. Much more could certainly be learned from the interac-

tion of integral membrane proteins with reverse micelles or similar membrane-mimetic structures, in experiments designed for this purpose.

Overall, the large body of published results also points to the exceedingly important role of water and water activity. It ranges from its function as a molecular lubricant bringing about the necessary flexibility to enzymes (Wu and Gorenstein, 1993), to more stringent requirements for refolding of catalytically active structures as described above in triosephosphate isomerase. Moreover, water is considered by Rand *et al.* (1993) almost as a variable part of protein structure. For example, these authors have shown that hexokinase releases at least 60 water molecules upon substrate binding. The evidence of such large dehydration-rehydration reactions has been brought to the attention of investigators by Colombo, Rau and Parsegian (1992). They imply significant contributions of different classes of water molecules to the energetics of conformational changes.

These findings open novel research routes in the field of proteins in reverse micelles where hydration can be finely tuned. Specifically conformational studies carried out at very low water content (Nicot *et al.*, 1985) and in relation with activity (Dorovska-Taran, Veeger and Visser, 1993b) should shed light on contribution of water to these mechanisms.

Finally one of the salient features of this review remains the existence of a gap between the increasing number of enzymes studied in reverse micelles and the scarcity of matching conformational data available from the literature. Additional and consistent studies are obviously required to further our knowledge on fundamental aspects of proteins and other characteristics of catalytic processes: in this respect proteins invited in reverse micelles behave as trouble-making and yet rewarding guests.

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