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Interactions of native and modified cytochrome C with a negatively charged reverse micellar liquid interface

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Abstract. Native and chemically modified cytochrome C were dissolved in sodium bis(2-ethylhexyl) sulphosuccinate (AOT)-oil-buffer microemulsions. The native cytochrome C contains 19 lysine residues, these groups were modified by 1) acetic anhydride or 2) succinic anhydride. At pH 8.4 the native, acetylated and succinylated proteins carry +8, −3 and −12 elementary charges, respectively. The phase behaviour of the microemulsion systems was found to be highly dependent on the charge of the proteins. Compared to a protein free system the native protein induces a L-2 phase separation at lower temperatures. The acetylated protein has a small effect on the temperature for the phase transition, whereas in the case of succinylated cytochrome C the phase transition takes place at higher temperatures. When dissolved in AOT microemulsions, the native cytochrome C has a perturbed tertiary structure, as indicated by loss of the 695 nm absorption band, while both the modified proteins retain the same optical properties when dissolved in an AOT microemulsion as in a pure buffer solution. The pertubed structure of the native cytochrome C was further investigated by testing the stability of the reduced form of the protein dissolved in the microemulsion media. The native cytochrome is unstable at $W > 10$, whereas the two modified proteins were found to be stable at all W-values investigated. The average location of the three proteins was determined by pulse radiolysis. The quenching rate constant of the hydrated electron depends upon the location of the probe in the reverse micelle; the succinylated protein is localised in the aqueous core of the reverse micelles, but both the native and the acetylated forms were found to be localised close to or at the AOT interface.

Key words: Microemulsion – Phase-behaviour – Protein – Interactions – Cytochrome C

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Introduction

A liquid interface can be formed by mixing surfactant, water and oil, where the surfactant forms a monolayer dividing the water and oil microdomains. Depending upon the composition and the physical parameters, the surfactant film can have very different topologies, varying from closed spheres to an interconnected 3D network. One intensively studied surfactant is sodium bis(2-ethylhexyl) sulphosuccinate (AOT). At low water contents this surfactant usually forms spheroidal aggregates called reversed micelles. These aggregates swell with water and, as the size of the aggregates increases, the concentration of discrete micelles decreases. Reverse micelles have been shown to serve as hosts for proteins and enzymes (Shield et al. 1986; Luisi et al. 1988; Martinek et al. 1989; Pileni 1989). One important parameter used to describe the reverse micelles is the water to surfactant ratio; $W = [H2O]/[AOT]$, and usually the properties of proteins in the microemulsion solvents are characterised as a function of the W-value. However, discrete droplet structure is not a requirement for enzyme catalysis in microemulsion solvents: bi-continous microemulsions have also been shown to serve well for enzymatic catalysis (Larsson et al. 1991).

Cytochrome C is a hemoprotein and its biological function is to transfer electrons in the respiratory chain of the mitochondras. It is a very well characterised protein, the amino acid sequence and the crystal structure are known (Dickerson and Timkovich 1975). Native cytochrome C from horse heart is a basic protein containing 19 lysine residues and at neutral pH the protein is highly positively charged. Most of the positive charges are situated in the electron-transfer interacting domain, whereas the negatively charged residues are placed on the opposite side of the protein molecule, which makes the protein a very strong dipole. The positive charges near the heme cleft are physiologically very important since the reaction partners of cytochrome C, as well as the inner mitochondrial membrane, have a negative charge. This gives reaction rates close to the diffusion limitation for the electron

transfer reaction (Margoliash and Bosshard 1983). Cytochrome C has been reported to have affinity for various negatively charged surfaces such as vesicles (Hildebrandt et al. 1990; Spooner and Watts 1991; Jongh et al. 1992) and proteins (Margoliash and Bosshard 1983; Lindsay et al. 1991).

Cytochrome C has been studied by several authors in reverse micelles (Balny and Douzou 1979; Dozou et al. 1989; Visser and Fendler 1982; Ermin and Metelitsa 1983; Pileni et al. 1985; Vos et al. 1987; Genkin et al. 1989; Brochette et al. 1988; Huruguen et al. 1991; Huruguen and Pileni 1991). There are indications that the positively charged cytochrome C interacts with the AOT surfactant layer (Pileni et al. 1985; Vos et al. 1987). A decrease of the micellar radius is reported if one molecule of cytochrome C is present per micelle as compared with a system without any protein (Brochette et al. 1988). Small angle X-ray scattering, pulse radiolysis (Pileni et al. 1985) and investigations of the fluorescence properties of a Zn derivative, all showed a strong interaction between cytochrome C and the AOT interface. However, in a microemulsion composed of the cationic surfactant CTAB, no interaction was observed (Vos et al. 1987). Native cytochrome C has also been reported to change the phase behaviour of an AOT/isooctane/water microemulsion. A percolation process was observed at lower temperature and at lower water volume fraction in the presence of cytochrome C as compared with unfilled droplets (Huruguen and Pileni 1991). Cytochrome C was also observed to induce strong attractive forces between the micelle droplets (Huruguen et al. 1991). These phenomena have been attributed to the location of the protein at the interface. In order to investigate the nature of these interactions we have, in this present investigation, made two derivatives of cytochrome C, which have a negative net charge, as compared to the native protein. This makes it possible to vary the net charge of the protein while keeping the pH constant. We observed that the charge of the protein has large effects on the phase behaviour, the percolation process, the protein folding and redox behaviour.

Material and methods

Chemicals

Cytochrome C Type VI from horse heart, succinic anhydride and AOT were purchased from Sigma and the organic solvents and borate acid (H_3BO_3) were bought from Fluka.

Preparation of the microemulsions

A stock solution of AOT-oil, filtered through a glass fiber filter (Whatman GF/A), was used to form the microemulsions with 25 mM borate buffer, pH 8.4. Isooctane, nonane or decane was added to give a constant final volume and an AOT concentration of 0.1 M. Borate buffer was used because of its compatibility with the pulse radiolysis experiments.

Phase behaviour

Samples were prepared by mixing a stock solution of AOT dissolved in oil, oil, and buffer containing the protein, in glass tubes equipped with stoppers. The tubes were kept in a thermostated water bath, the temperature was varied and the stability region of the microemulsion phase was determined visually. To detect any lamellar liquid crystalline phase, the samples were observed between crossed polarisers.

Conductivity measurements

The samples were thermostated in a water bath and the conductivity was measured using a Tacussel conductimeter, type CD 810.

Pulse radiolysis

A Febetron 707 was used to generate electrons. 20 ml of the sample was degassed by bubbling with argon. Typical intensities were in the range of 300–500 mV. The decrease of the hydrated electron absorption was observed for around 800 ns, at 720 nm for the native cytochrome C, and at 750 nm for the modified proteins. This higher wavelength for the modified proteins was preferred because these proteins retain the absorption band centered at 695 nm in the AOT microemulsions. The quenching was analysed using a non-linear least-squares fitting (Pileni et al. 1984).

Cytochrome C modification and characterisation:

Protein modification

Ferricytochrome C was acetylated and succinylated by the method of Finkelstein et al. (1981). The protein was dissolved in a half-saturated solution of sodium acetate or disodium succinate at 0 °C. Under stirring a five times molar excess (referred to the number of lysine groups) of acetic/succinic anhydride was added in portions. After 30 minutes the reacting solutions were dialysed (spectrapore, mwco 6 000–8 000) against several exchanges of millipore water at 4 °C and the pH was adjusted to 7. The protein solutions were concentrated before lyophilization using a Diaflo ultrafiltration unit equipped with a PM10 membrane. The cytochrome C solutions were further washed, using the ultrafiltration unit, with millipore water until the eluate showed a low and constant conductivity.

HPLC

HPLC separation was performed on a strong anion exchanger column, TSK-Gel DEAE-5PW (15 cm × 21.5 mm ID). A Waters 600E system equipped with a Waters 991 detector system was used for the analyses.

Table 1. Charge density of native and modified cytochrome C at pH 8.4

Group	pKa	number	Native	Acetyl	Succinyl
Terminal COO^-	2,0	1	−1	−1	−1
Asp, Glu	4,5	12	−12	−12	−12
His	6,0	2	0	0	0
Lys	10,5	19	+19	+7,8	+9
Arg	12,5	2	+2	+2	+2
Succ-COO^-	4,5	–	–	–	−10
Net charge			+8,0	−3,2	−12

The column was equilibrated and the proteins dissolved in 20 mM Tris-HCl buffer, pH 8.15. The proteins were eluted using a linear gradient of the same buffer containing 0-0.4 M NaCl in 60 minutes. Under the conditions employed, any native cytochrome would elute with the dead volume, whereas the acetylated and succinylated species elute at 35 and 50 minutes after injection, respectively. Both preparations were found to be almost completely free of any native protein.

Protein average charge

The theoretical charge of the native cytochrome C was calculated from the chemical composition (Dickerson and Timkovich 1975), assuming the pK_a values indicated in Table 1 and that all polar amino acids are situated on the surface. The heme propionate groups give no contribution to the charge since they are deeply buried within the protein matrix (Buchnell et al. 1990) and the tyrosine groups are assumed to be non-dissociated.

The degree of modification was determined by titration of the numbers of free $-NH_2$ groups using an OPA reagent (Church et al. 1985). The cytochrome C was mixed with the reagent solution to give a protein concentration of 0–20 μM. After 2 minutes incubation, the absorbance was measured at 335.5 nm. From the slope and using the literature value of the extinction coefficient, $\varepsilon = 6\,000\ M^{-1}cm^{-1}$, the residual numbers of $-NH_2$ groups was deduced. Using this value the charge of the modified proteins was calculated. At pH 8.4 the acetylated protein was found to have a net charge of approximately −3 and the succinylated form −12, whereas the native cytochrome had a positive net charge of 8 (these values are given in Table 1).

Spectrophotometric characteristics

In aqueous solution, the absorption spectrum of native cytochrome C is well known and characterised by a Soret band centred at 409 nm, q bands around 550 nm and a weak absorption peak centred at 695 nm. The spectral properties of the modified proteins were found to be almost indistinguishable from those of the native protein, when solubilized in the borate buffer, in the range 300–750 nm. The only difference to be noted is a small shift in the maximum of the Soret-band for the acetylated form 407 instead of 408 for the native cytochrome.

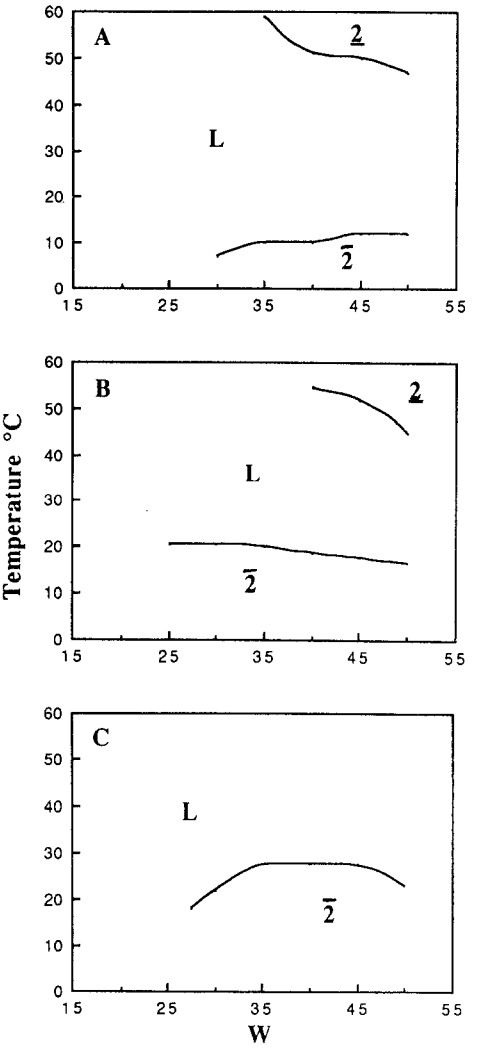

Fig. 1. Phase behaviour of 0.1 M AOT-isooctane-25 mM borate buffer containing, as referred to the overall volume, 0.2 mM of native **A**, acetylated **B** and succinylated **C** cytochromes C, respectively. L denotes the homogenous microemulsion region, 2 the two phase system with excess water in equilibrium with a microemulsion and 2 the two phase system with excess oil in equilibrium with a microemulsion phase

The protein concentration was determined by reading the absorbance at 550 nm for the reduced cytochrome C, using the corresponding extinction coefficient, $\varepsilon = 27\,700\ M^{-1}cm^{-1}$. The protein was totally reduced by a few grains of dithionite.

Results and discussion

Changes of the microemulsion phase behaviour in the presence of native and modified cytochrome C:

The phase behaviour of the native cytochrome C in AOT/isooctane microemulsions is illustrated in Fig. 1 A as a function of the water content, $w = [H_2O]/[AOT]$. The isotropic microemulsion phase, represented by L, is stable at ambient temperatures. At temperatures below the microemulsion region there is a two-phase region where

the microemulsion is in equilibrium with excess water (2). At higher temperatures one observes a two-phase region consisting of a microemulsion in equilibrium with excess oil (2).

Using the chemically modified cytochromes one observes a major change in the phase behaviour as compared with a system containing the native cytochrome C. The schematic phase diagram is given in Fig. 1 B for the acetylated cytochrome and in Fig. 1 C for the succinylated species. Using isooctane as oil and 0.2 mM of the protein derivatives it is not possible to obtain a microemulsion phase for all three proteins at room temperature. This causes a problem when one wants to compare the three proteins using an equivalent microemulsion solvent. However, a shift in the phase behaviour can be obtained by changing the oil of the continuous phase (Kunieda and Shinoda 1980); for an ionic surfactant a microemulsion can be formed at lower temperatures as the length of the hydrocarbon increases. In Fig. 2 the phase behaviour of AOT-brine-decane system is presented without protein and containing native, acetylated and succinylated cytochrome C. As can be seen, the microemulsion phase is observed at lower temperatures as compared with the system composed of isooctane. Also, the relative position of the phase boundary changes for the different proteins added to the system. The native cytochrome C induces a phase transition at lower temperatures when compared with a system without protein. The acetylated protein hardly affects the temperature for the phase transition, whereas for the succinylated protein the phase transition is found at higher temperatures.

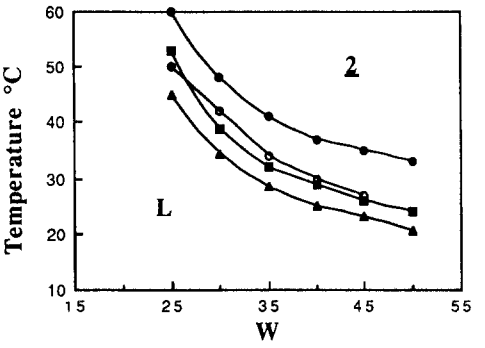

Fig. 2. Phase behaviour of 0.1 M AOT-decane-25 mM borate buffer without protein (m) and containing 0.2 mM of the native (▲), acetylated (□) and succinylated (♦) cytochrome C as referred to the overall volume. Notations as above

Percolation processes observed using filled and unfilled microemulsions:

As the temperature in the one-phase microemulsion region is varied, keeping W constant, the microstructure changes (Chen et al. 1990). The temperature changes the solubility of the AOT surfactant. With increasing temperature the AOT becomes more hydrophilic. At low temperature close to the 2-phase border, water-in-oil droplets are formed, and as the temperatures increases, these droplets cluster and open up further a form a 3D network. This change of the microstructure, sometimes referred to as percolation of the system, can be followed by electrical conductivity measurements (Chen et al. 1990). The percolation onset can be changed by different additives. It has been demonstrated that the solubilisation of high concentrations of native cytochrome C (4 proteins per water droplet) favours the percolation process with an increase in the conductivity at lower polar volume fraction and temperature (Huruguen and Pileni 1991). Similar behaviour is also observed for lower native cytochrome C concentrations as shown in Fig. 3 A (0.4 mM cytochrome corresponds to 3 proteins per water droplet, at $W = 40$ and 0.1 M AOT). Here the conductivity is plotted as a function of the temperature. As the native cytochrome C concentration increases, the percolation onset is induced at lower temperatures. The behaviour of the microemulsion containing the acetylated and succinylated cytochrome C shows the opposite behaviour as compared with the case of native cytochrome C. As seen in Figs. 3 B and 3 C the percolation is observed at higher temperatures as the protein concentration increases, with one exception for the acetylated protein at 0.4 mM that showed the same critical temperature for the percolation onset as the system without any protein. A simple salt effect could explain the data observed for the modified cytochrome C. In Fig. 3 D the effects on the percolation of sodium chloride are illustrated, the percolation onset is shifted to higher temperature when the salt concentration increases. However, a simple salt effect cannot explain the behaviour observed when using native cytochrome C, where the temperature of percolation onset decreases with increasing cytochrome C concentration (Fig. 3 A). From Fig. 3 A one can note that the maximal conductivity before phase separation gets lower with increasing cytochrome concentration. This behaviour could be explained either by the presence of the strong dipole moment (up to 300 Debyes) of native cytochrome C inducing dipole-dipole interactions between droplets or by the hydrophobic character of the protein favouring the interconnection between the microphases or by a change head-group area of the surfactant.

In Figure 4 the behaviour of an AOT/brine/decane microemulsion containing either of the three proteins is compared to a protein free microemulsion (indicated at 0 in the graph). The temperatures for the phase transition and the percolation onset are given as a function of the charges of the proteins. This figure confirms again that the percolation onset, as compared to a protein free system, is reached at lower temperatures for the native cytochrome C and at higher temperatures for the two modified cytochromes. One remarkable observation is that the temperature range in which the system is highly conducting, varies for the different systems: for the microemulsion containing the native and acetylated protein this range is 3.5 °C, and for the empty and the succinylated protein the interval is 6 degrees.

Spectroscopic characteristics of native and modified cytochrome C in microemulsions

The UV-VIS spectrum of cytochrome C can give information about the protein conformation. Horse ferricy-

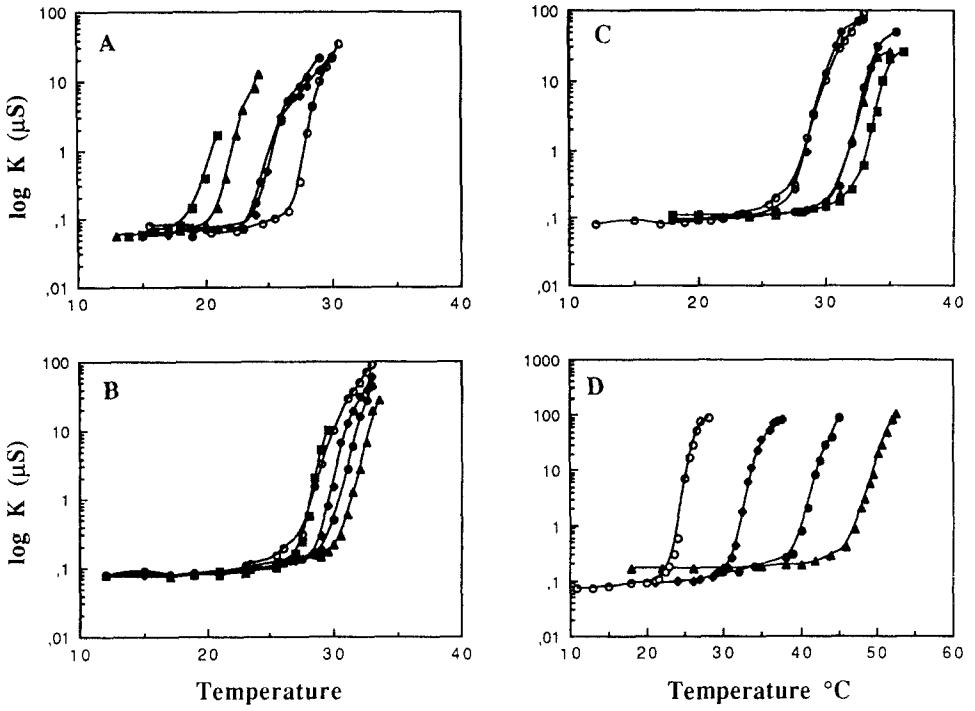

Fig. 3. Variation of the conductivity as a function of the temperature for a microemulsion composed of 0.1 M AOT-decane-25 mM borate buffer at W = 40, containing various concentrations referred to the aqueous phase of: **A** native; **B** acetylated; **C** succinylated cytochrome C: 0 mM (m); 0.1 mM (♦); 0.2 mM (|); 0.3 mM (▲) and 0.4 mM (n) protein; **D** NaCl: 0 mM (m); 25 mM (♦); 50 mM (|); 75 mM (▲)

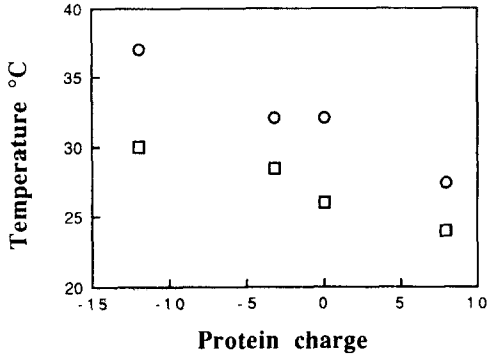

Fig. 4. Percolation (p) and phase transition (m) temperatures as a net charge of the proteins in a microemulsion composed of 0.1 M AOT-decane-25 mM borate buffer, at W = 40 using 0.2 mM of the cytochromes. The values at 0 indicates the protein free system

Table 2. Spectral properties of native and modified cytochrome C in buffer and in AOT microemulsions, Wo = 20

Cytochrome C	Solvent	Heme	695-band
Native	Borate buffer	408	Present
	AOT	407	Absent
Succinylated	Borate buffer	408	Present
	AOT	408	Present
Acetylated	Borate buffer	407	Present
	AOT	407	Present

tochrome C is known to proceed through stages of denaturation, all with different absorption characteristics (Drew and Dickerson 1978):

i) Part of the polypeptide uncoupled from the heme, this gives a shift of the Soret band from 409 to 407 nm.

ii) Displacement of the methionine ligand from the heme iron, this leads to the disappearance of the 695 nm band.

iii) Protonation and displacement of the histidine ligand, this is associated with a shift of the Soret band to 395–400 nm and the appearance of a new band at 620 nm.

The spectral properties of native cytochrome C in AOT-water-isooctane microemulsions have been shown to be different as compared to those for the protein dissolved in pure buffer (Douzou et al. 1979; Visser et al. 1982; Brochette et al. 1988). At low hydration degree, W < 15, the absorption of the Soret band is blue shifted with an increase of its intensity. The CD spectra of native cytochrome C in AOT reverse micelles have shown that the helical structure is partly lost, whereas in CTAB microemulsions no perturbation was observed (Vos et al. 1987). Furthermore, the 695 nm absorption band due to heme-bound methionine was also lost when solubilizing cytochrome C in an AOT microemulsion (Brochette et al. 1988). The change of the absorption spectrum at low water content can be attributed to an increase in the interactions between the polypeptide and the heme. The disappearance of the 695 nm band and the changes in the CD spectra indicate that the native cytochrome C is in a non-native configuration in AOT reverse micelles.

Our experimental data confirms the perturbations of the spectroscopic properties of native cytochrome C dissolved in AOT microemulsions. Table 2 represents the maximum for the Soret band and the absence or presence of the 695-band for the native, acetylated and succinylated cytochrome C dissolved in buffer and in an AOT microemulsions. For the native protein a blue shift is observed for the Soret band and no absorption is found at

695 nm, whereas in the case of the acetylated and succinylated cytochromes, the absorption spectra are unchanged as compared with those obtained in aqueous solution as characterised by the Soret and q bands and normal 695 nm band. This indicates that the modified, negatively charged, cytochromes do not interact with the interfacial wall of the AOT reverse micelles.

Redox-activity of native and modified cytochrome C in microemulsion

In an aqueous solution the Fe^{2+} ion is oxidised if O_2 is present. However, in cytochrome C the polypeptide protects the Fe^{2+} ion from the surrounding solvent and the reduced native cytochrome C remains stable. The stability of the reduced form of the native cytochrome has been tested when dissolved in microemulsions. The protein was reduced by sodium dithionite and dialysed; the reduced protein was injected into a microemulsion and the absorbance at 550 nm was followed. The results are given in Table 3 as a function of the W value of the microemulsions. For the native cytochrome the reduced form is only stable at W = 10, whereas for the two modified proteins the reduced form is stable at all W values investigated. This is most probably explained by a destabilised tertiary structure of the native cytochrome induced by the electrostatic interactions between the positively charged protein and the negatively charged interface. This is also confirmed by the fact that native cytochrome C loses its 695 nm absorption band in AOT reverse micelles. Whereas for the two negatively charged cytochromes, the proteins are repelled from the negative AOT and confined inside the water pool of the droplets.

Average location of cytochromes determined by pulse radiolysis:

To further confirm these results the average locations of the proteins dissolved in the reverse micelles were examined by kinetic measurements using hydrated electrons developed in the laboratory (Pileni et al. 1984; Petit et al. 1986). From the reaction rate constant, k_q, of the hydrated electrons with a probe solubilized in a reverse micelle, the average location of the probe can be deduced, assuming that the symmetry of the micelles is spherical and the intra-micellar exchange is slower than the quenching process. The model is confirmed by Gostle et al. (1986) who suggested that the rate of the quenching depends on r^{-3} for a probe located in the aqueous core of the micelle and on r^{-2} if the probe is located at the interface. As the water pool radius, at $W < 50$, is directly proportional to W (Pileni et al. 1985) one expects the quenching rate constant, k_q, to vary with W^{-3} or W^{-2}, depending on the location of the probe.

The pulse radiolysis experiments require a high protein concentration and, as discussed above, the increase in cytochrome concentration induces changes in the phase behaviour. Because of this, different oils were used for the three proteins. Isooctane was used for the native cytochrome C, nonane for the acetylated and decane for the succinylated protein. All samples investigated have a low conductivity, which indicates a droplet structure. In Fig. 5 A–C, log k_q versus log W is plotted for the native, acetylated and succinylated cytochromes. The slope for the native and acetylated cytochromes was found to be −2.2, that for the succinylated protein was −2.9. The scatter of the experimental points is due to changes in the electron intensities. Here all the data points are given and the mean values were used to calcu-

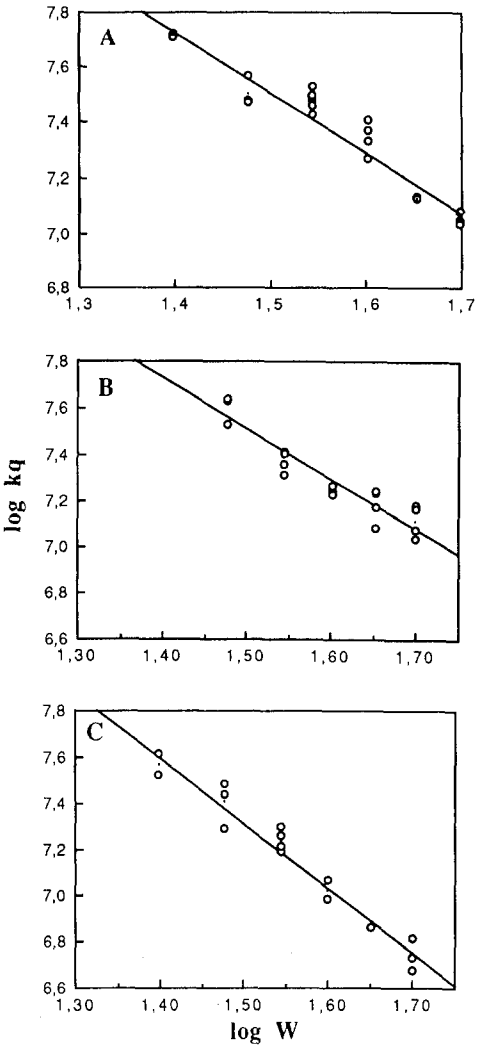

Fig. 5 Variation of the quenching rate constant for hydrated electrons as a function of the water content in the presence of 0.2 mM native **A** acetylated **B** and succinylated **C** cytochromes C respectively

Table 3. Stability of the reduced form of cytochrome C in AOT microemulsions

Cytochrome C	Wo = 10	Wo = 20	Wo = 30	Wo = 40
Native	Stable	Unstable	U	U
Succinylated	S	S	S	S
Acetylated	S	S	S	S

late the slopes. The experimental errors can be estimated to be less than 15%. However the consistency of the results was verified by repeating the experiments and obtaining very similar results.

These results confirm the strong perturbation of the spectral and activity data for the positively charged native cytochrome, indicating that the protein is situated at the AOT interface, whereas the negatively charged succinylated cytochrome C is dissolved in the aqueous core of the reverse micelle. The result for the acetylated protein can be explained by the fact that the electron-transfer interaction domain is still positively charged, though with a lower charge density. There might also be another type of hydrophobic interaction. However, since the acetylated protein retains the native spectral properties and is also stable in the reduced form, the tertiary structure of the protein must be well preserved when incorporated into the micellar solvent.

Conclusions

Native, positively charged, cytochrome C is strongly associated with the negatively charged AOT interface: this induces mutual changes of both the protein folding and the microemulsion phase behaviour. The perturbed tertiary structure of cytochrome C changes the spectral and redox properties of the protein. This is further supported from the literature where conformational changes are found to be induced by electrostatic interactions with various negatively charged surfaces such as phospholipid vesicles (Hildebrandt et al. 1990) and cardiolopin bilayers (Spooner and Watts 1991). Structural changes of cytochrome C in AOT micelles are also found using resonance Raman spectroscopy (Hildebrandt and Stockburger 1989). Part of the α-helix structure was shown to be lost when the native protein was mixed with negatively charged direct micelles (Jongh et al. 1992). The presence of the native cytochrome C also changes the behaviour of the AOT interface. The surfactant headgroups are probably forced to a less dense packing, which gives a lower mean curvature to the surfactant film. This is reflected in a strong preferance of an interconnected system to form discrete micelles; hence the temperature for the phase separation is decreased. If on the other hand, the protein is converted to a highly negative polyelectrolyte, the effects in an AOT microemulsion will be the same as for a monovalent salt such as NaCl, and the protein will retain the same properties as in a pure buffer solution.

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