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Ligand binding at membrane mimetic interfaces

Human serum albumin in reverse micelles

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The behaviour of human serum albumin in the presence of three chemically distinct ligands: oxyphenylbutazone, dansylsarcosine and hemin, has been compared in buffer and in reverse micelles of isooctane, water, and either sodium bis(2-ethylhexyl)sulfosuccinate or hexadecyl trimethylammonium bromide, systems selected to mimic the membrane-water interface. Upon micellar incorporation, the dansylsarcosine-albumin complex dissociated, as evidenced by fluorescence emission spectroscopy (red shift from 485 nm to 570 nm) and by fluorescence polarization measurements. In contrast, the hemin-albumin complex remained stable in reverse micelles, as judged from the Soret absorption band at 408 nm and the molar absorption coefficient of 8.4×10^4 M^{-1} cm^{-1}. The oxyphenylbutazone to albumin binding curves reveal that while the association constant remained unchanged ($K_a \approx 1.0 \times 10^5$ M^{-1}), only a fraction of the albumin molecules present reacted with the ligand. The results were unaffected by the nature and the concentration of the surfactant.

These findings can be interpreted in the light of conformational changes induced in human serum albumin by the large micellar inner surface area. The blue shift of the fluorescence emission maximum from 344 nm in buffer to 327 nm in sodium bis(2-ethylhexyl)sulfosuccinate micelles and the lesser reactivity/accessibility of the fluorophore to oxidation by *N*-bromosuccinimide, indicate perturbations of the sole tryptophan-214 microenvironment. However, the distance between the indole residue and tyrosine-411 does not seem substantially modified by the 15% decrease affecting the α helices of the albumin molecule. It is proposed that the results reported herein reflect the interactions of albumin with a membrane-like interface which generates two protein subpopulations differing in their membrane-surface and ligand affinities. Overall and local conformational changes, originating from this surface-induced effect, may thus constitute a ligand-release facilitating mechanism acting at cellular membrane levels.

The use of organized microassemblies of surfactants, water, and apolar solvents for incorporation of enzymes or proteins active at interfaces is now well documented (Martinek et al., 1986; Luisi and Magid, 1986; Luisi et al., 1988). Once incorporated into reverse micelles, the guest macromolecules may display changes in conformation (Waks, 1986; Luisi and Steinmann-Hoffmann, 1987) induced by interactions between the charges of the surfactant polar head groups and those of the protein (Nicot et al., 1985; Petit et al., 1986; Gallay et al., 1987), and they may acquire characteristic alterations of their biological functions (Klyachko et al., 1986). Less attention has been given to the behavior of another important category of macromolecules: the drug transport proteins. Human serum albumin (HSA), for example, is the carrier of a large array of hormones and therapeutic drugs (Kragh-Hansen, 1981) which have to be released and delivered to the tissues.

The degree to which a ligand is bound to a plasma transport protein controls its delivery to cells. Recent findings (Weisiger et al., 1989) have led to the reevaluation of the respective roles of free and albumin-bound ligands in transport, which has been reported to correlate better with the albumin-bound than with the free concentration (Forker and Luxon, 1981; Pardridge, 1988). Finally, preliminary studies of the three-dimensional structure of HSA crystallized in microgravity experiments (Carter et al., 1989) have renewed interest in these questions, although the existence of an albumin receptor remains a subject of conjecture and controversy (Weisiger et al., 1981; Wolkoff, 1987).

In this respect, a recent paper by Reed and Burrington (1989) has provided convincing evidence for a surface-induced conformational change in HSA, which gives rise to two subpopulations of albumin with different cell-surface affinities and may thus mimic a receptor effect. The question of a simultaneous ligand-release-facilitating mechanism was not addressed by the authors.

Since interfacial phenomena and surface/protein interactions play a key role in the organization and control of biological membrane mechanisms, we have been prompted to explore the possible perturbations induced by micellar incorporation of HSA in the protein conformation, as well as in the transport function itself. Because the anisotropic and amphipatic nature of biological membranes is preserved to a large extent by the

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Abbreviations. FITC, fluorescein isothiocyanate isomer I; FRAPP, fluorescence recovery after fringe photobleaching pattern; HSA, human serum albumin; K_a, association constant; R_H, hydrodynamic radius; W_o, molar water/surfactant ratio.

reverse micellar system (Nicot et al., 1985) the very large interfacial surface generated by the reverse micellar system is expected to amplify such effects.

In order to pursue the question, we have incorporated HSA into reverse micelles of anionic and cationic surfactants, water and isooctane. Fluorescence emission spectroscopy and circular dichroism have been employed to detect the resulting alterations of albumin structure. The reactivity of the lone tryptophan residue (Trp214) has been probed by oxidation with *N*-bromosuccinimide. The perturbation of the ligand-binding function of the protein upon micellar incorporation was measured with dansylsarcosine, oxyphenylbutazone and hemin using spectroscopic techniques. The results obtained are interpreted in the light of the findings of Reed and Burrington (1989). They underline the importance of the membrane interface in the facilitation of a ligand-HSA release mechanism.

MATERIALS AND METHODS

Chemicals

Sodium bis(2-ethylhexyl)sulfosuccinate, a generous gift of Cyanamid Co. (France), was purified according to Wong et al. (1976) and dried. Hexadecyltrimethylammonium bromide was obtained from Serva (Heidelberg, FRG), tetra-(ethyleneglycol) mono-*n*-dodecyl ether was from Nikko Chemical Co. (Tokyo, Japan). Isooctane 'Uvasol' spectroscopic grade and 1-hexanol were purchased from Merck (Darmstadt, FRG). Human serum albumin (crystallized, fatty-acid- and globulin-free), dansylsarcosine piperidine salt, crystalline hemin of type II, *N*-bromosuccinimide, oxyphenylbutazone, fluorescein isothiocyanate isomer I (FITC), *p*-nitrophenyl anthranilate were obtained from Sigma Chemical Co (St Louis, MO, USA). BioGel P2 was from BioRad (Richmond, CA, USA). All other reagents were analytical grade or the best available.

Preparation of reverse micelles

The micellar solutions were prepared by addition of measured volumes of isooctane to dry, preweighed amounts of sodium bis(2-ethylhexyl)sulfosuccinate. The required volume of protein solution in the appropriate buffer was injected with a Hamilton syringe into the surfactant solution. For hexadecyltrimethylammonium bromide reverse micelles the protein solution was injected onto the mixture of hexadecyltrimethylammonium bromide/isooctane/hexanol (8–12% by vol.). The samples were shaken until optically clear. The final solutions were ultrafiltered or centrifuged to remove any undissolved material. The water/surfactant molar ratio was defined as $W_o = [H_2O]/[\text{surfactant}]$.

Since micellar solutions are optically transparent, the albumin concentration was determined spectrophotometrically using a molar absorption coefficient of $3.5 \times 10^4\ M^{-1}\ cm^{-1}$ at 278 nm in both buffer and micellar solutions. Absorption measurements were carried out on a Cary 118 or a Uvikon 860 spectrophotometer.

Oxyphenylbutazone binding studies

The binding of oxyphenylbutazone by albumin was measured either by absorption or by fluorescence emission spectroscopy (Maes et al., 1979). In the first case, the addition of successive increments of oxyphenylbutazone (0.3–0.5 mM) to 2 ml HSA (0.015–0.020 mM), in 0.1 M Tris/HCl pH 8.3, generated a sequential increase of the absorbance at 278 nm, until a plateau value was reached. In the second case, the fluorescence emission spectrum of HSA (1.0 μM) was recorded with excitation at 295 nm. Upon each addition of the drug (0.1 mM), the decrease of the maximum intensity in the 340–350-nm range was determined. All the spectra were corrected for dilution and self-absorption (Lakowicz, 1983). Identical measurements were carried out with HSA in micellar solutions of sodium bis(2-ethylhexyl)sulfosuccinate, hexadecyltrimethylammonium bromide or tetra(ethyleneglycol) mono-*n*-dodecyl ether in organic solvents all at $W_o = 22.4$. The protein was titrated with the corresponding micellar solutions of oxyphenylbutazone. Drug titrant solutions all contained sufficient HSA to maintain constant protein concentration throughout a titration.

Preparation of the albumin-ligand complexes

Dansylsarcosine-HSA. Equal volumes of 0.30 mM HSA and 0.22 mM dansylsarcosine solutions in 0.1 M borate pH 8.0 were mixed dropwise in the cold and kept in the dark for several hours. A volume of 36 μl of the resulting complex was injected either into 1 ml of the same borate buffer or into 1 ml of a 0.1 M sodium bis(2-ethylhexyl)sulfosuccinate solution in isooctane in stoppered cells. This corresponds to a final W_o value of 22.4.

Hemin-HSA. Hemin was first dissolved in 0.1 M NaOH. The stock solution (11 mM) was diluted in a 0.1 M borate pH 8.0 and added dropwise to a solution of albumin in the same buffer with slow stirring at 4 °C. The final concentration of the mixture was 0.38 mM albumin and 0.49 mM hemin; the pH of the buffered solution was 8.25. The reaction was allowed to proceed for 1 h. The resulting mixture (0.5 ml), with a heme/protein molar ratio of 1.2, was layered on a P2 column (0.6 × 6.5 cm) equilibrated in the same borate buffer to separate unbound hemin from the hemin-HSA complex. The complex was injected with a Hamilton syringe into buffer or into a micellar solution, as described above.

Chemical modifications of albumin

Anthraniloyl labeling of HSA. The anthraniloyl derivative was prepared according to Hagag et al. (1983). The excess reagent was removed on a P2 column equilibrated with 0.1 M phosphate pH 7.4.

FITC labeling. FTC-albumin was obtained as described in detail by Chatenay et al. (1987a) and incorporated into reverse micelles of 0.03, 0.05 and 0.1 M sodium bis(2-ethylhexyl)sulfosuccinate, W_o 22.4, 0.1 M Tris pH 8.0. The absorbance of micellar solutions of FTC-HSA was kept between 0.06–0.12 at 495 nm. The FTC/HSA molar ratio was 0.65 ($\varepsilon = 72.1\ mM^{-1}\ cm^{-1}$ at pH > 8.1; Garel, 1976).

Oxidation of the albumin tryptophan by N-bromosuccinimide. The reaction of *N*-bromosuccinimide with Trp214 was carried out in aqueous and micellar solutions as previously described by Nicot et al. (1985). The course of tryptophan oxidation reaction was followed by the decrease in fluorescence emission (Privat et al.,1976). HSA was excited at 295 nm, and the emitted spectrum was recorded in the 300–400-nm range. When the indole oxidation was completed, the protein solution was excited at the tyrosine maximum emission wavelength (280 nm) to check the integrity of the phenolic side-chains.

Fluorescence measurements

Corrected fluorescence emission spectra (3–5 nm bandwidth) were recorded on a Hitachi 4010 spectrofluorometer at 20 °C. In all spectra presented the buffer baseline was substracted, eliminating a constant background and the water Raman peak at 300 nm. Fluorescence polarization measurements were carried out on an SLM model XE 450 apparatus (SLM Instruments, Urbana, IL, USA). The temperature of the cell housing was kept at 20 °C with a circulating water thermostat.

Fluorescence recovery after fringe pattern photobleaching (FRAPP)

This technique allows measurement of the self-diffusion coefficient D_S of fluorescent probes. When strongly illuminated, these probes irreversibly lose their fluorescent properties (photobleaching). In the illuminated volume the fluorescence intensity is measured afterwards by use of a low-intensity light beam. The signal increases as new probes enter the studied volume and D_S is deduced from the recovery time. The setup has been described previously (Chatenay et al., 1987b). The recovery curves were fitted to the expression:

$$I(t) = Ae^{-t/\tau} + B$$

where the characteristic time of fluorescence recovery $\tau = i^2/4\pi^2 D_S$ and i is the fringe spacing. For each sample, we verified that τ^{-1} scaled with $q^2 = (2\pi/i)^2$ and D_S was obtained from the τ^{-1} versus q^2 plot. Typical fringe spacings were in the 10–100-μm range.

Circular dichroic spectroscopy

The HSA solutions used for CD spectroscopy were 17–18 μM. 0.05 M Tris pH 8.0 was used for buffer solutions of the protein as well as for the aqueous phase in reverse micelles, which were 100 mM in sodium bis(2-ethylhexyl)sulfosuccinate at $W_o = 22.4$. Spectra were recorded on an Aviv model 60 DS CD spectrometer in cylindrical cells. Near and far ultraviolet spectra were taken for HSA in buffer an in micelles. Baselines were scanned in the same cells as were used for the protein, care being taken to place the cells in the same orientation for all scans. Cells of 1 cm path length were used in the near ultraviolet (250–330 nm), while in the far ultraviolet (207–250 nm) a cell of path 0.1 cm was used for HSA in buffer. For HSA in reversed micelles in the far ultraviolet the path length was 0.05 cm. The near and far ultraviolet in buffer and the far ultraviolet in micellar solution were scanned three times each, while the near ultraviolet in micelles was scanned six times to reduce noise. Solvent baselines were substracted after signal averaging of the sample and baseline solutions and the results converted to mean residue ellipticities.

RESULTS

Solubilization of HSA in reverse micelles

The protein was easily solubilized in micellar solutions of 0.1 M anionic [sodium bis(2-ethylhexyl)sulfosuccinate], cationic (hexadecyltrimethylammonium bromide) or non-ionic [tetra(ethyleneglycol) mono-*n*-dodecyl ether] surfactants in variable amounts of water expressed as $W_o = [H_2O]/[\text{surfactant}]$. At W_o values between 20–30 the incorporated protein concentration varied in the range 1.5–400 μM. The ultraviolet absorption spectra (not shown) in buffer (0.1 M Tris/HCl pH 8.3) or in reverse micelles are virtually identical. Lack of scattering in the 350–450-nm region indicates excellent solubilization of the protein.

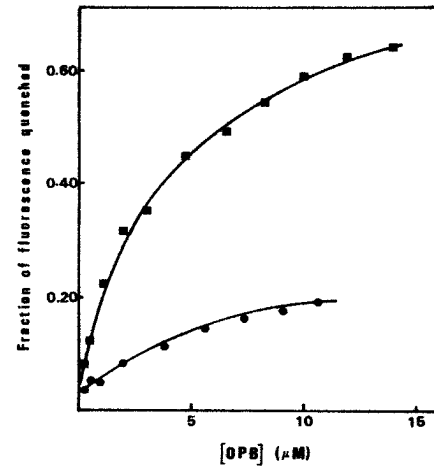

Fig. 1. *Fluorescence quenching titration curves of oxyphenylbutazone (OPB) binding to HSA.* (■) In 0.1 M Tris pH 8.0; (●) in reverse micelles of 50 mM sodium bis(2-ethylhexyl)sulfosuccinate, $W_o = 22.4$, in the same buffer. 2 ml 1.4 μM HSA was titrated by successive increments of 0.1 mM oxyphenylbutazone containing 1.4 μM HSA

The fluorescence emission spectrum ($\lambda_{exc} = 295$ nm) of the single HSA Trp214 residue was recorded in buffer and in reverse micelles. In aqueous solutions the emission maximum was 344 nm, while the fully exposed tryptophan of *N*-acetyltryptophanamide had its maximum at 354 nm. In reverse micelles the fluorescence intensity is decreased by about 30%, and all the emission maxima are shifted to the blue: 337 nm for tetra(ethyleneglycol) mono-*n*-dodecyl ether, 330 nm for hexadecyltrimethylammonium bromide, 327 nm for sodium bis(2-ethylhexyl)sulfosuccinate. These values were unaffected by the volume of water present in the W_o range 11.2–40 or by the surfactant concentration.

Binding of oxyphenylbutazone to albumin

The HSA fluorescence emission maximum is quenched by titration with oxyphenylbutazone. The quenching $Q = 1 - F/F_o$ where F_o and F are the corrected fluorescence values for the free and bound protein solution. The fluorometric titration curves, in which Q is plotted against the total oxyphenylbutazone concentration, seem to follow a simple hyperbolic saturation isotherm. The major difference between the two binding curves (Fig. 1), carried out at identical concentrations of reactants, consists in the quenching values reached at saturating oxyphenylbutazone values. In the aqueous solution the quenching reaches 0.7, while in reverse micelles it levels off at only 0.2. The same type of binding curves was observed by absorption spectroscopy and with the different surfactants used in this study.

Determination of the oxyphenylbutazone binding constants

It is assumed that the fluorescence (or the absorption) change is directly proportional to the concentration of oxyphenylbutazone-HSA complex. The molar fraction, R, of the protein bound at each concentration of oxyphenylbutazone is given by:

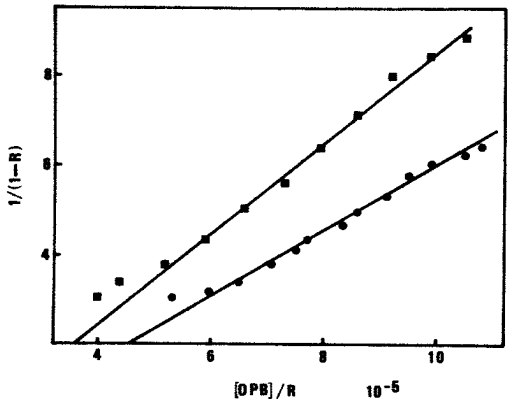

Fig. 2. *Typical plot of* $1/(1-R)$ *versus* $[oxyphenylbutazone]_{total}/R$. R is the molar fraction of HSA bound to oxyphenylbutazone: (■) in 0.1 M Tris pH 8.0; (●) in reverse micelles of 50 mM sodium bis(2-ethylhexyl)sulfosuccinate $W_o = 22.4$. K_a was obtained as the reciprocal of the slope of the curve (see Table 1)

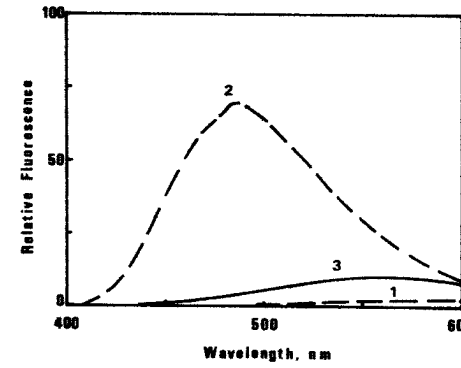

Fig. 3. *Fluorescence emission spectra of dansylsarcosine and dansylsarcosine-albumin complexes* ($\lambda_{exc.} = 337$ *nm*). The fluorescence intensity is expressed in arbitrary units. Curve 1, dansylsarcosine in 0.1 M borate pH 8.0; curve 2, dansylsarcosine-albumin complex 8 μM, in buffer; curve 3, dansylsarcosine and dansylsarcosine-albumin complex in reverse micelles 0.1 M sodium bis(2-ethylhexyl)sulfosuccinate, $W_o = 22.4$

Table 1. *Binding parameters of oxyphenylbutazone to albumin*
All experiments were carried out in 0.1 M Tris pH 8.0 at 20°C. In micellar solutions the water/surfactant molar ratio $W_o = 22.4$. The results are average values with the number of experiments in parentheses. Total albumin concentration used: 20 − 1.4 μM

Medium	$10^5 \times K_a$ (n)
	M^{-1}
Buffer	2.34 ± 0.6 (5)
Sodium bis(2-ethylhexyl)sulfosuccinate reverse micelles	1.70 ± 0.6 (4)
Hexadecyltrimethylammonium bromide reverse micelles	2.15 (2)

$$R = [\text{HSA-oxyphenylbutazone}]/[\text{HSA}]_t = \Delta F/\Delta F_{max} = \Delta Q/\Delta Q_{max}$$

where ΔQ_{max} is the maximum quenching at saturation, ΔQ is the observed quenching change and $[HSA]_t$ = the total protein concentration. ΔQ_{max} can be calculated from a linear reciprocal plot (Q change vs 1/[oxyphenylbutazone]. K_d, the dissociation constant, and oxyphenylbutazone binding site concentration are obtained from the replot of $1/(1-R)$ vs $[oxyphenylbutazone]_t/R$ according to the Gutfreund (1972) equation:

$$1/(1-R) = [\text{oxyphenylbutazone}]_t/(K_d R) - [\text{HSA}]_t/K_d.$$

Plots of $1/(1-R)$ versus $[oxyphenylbutazone]/R$ were linear (Fig. 2). At high protein concentration ($\gg K_d$) accurate information is gained about the concentration of binding sites, while at low protein concentration ($\ll K_d$) accurate information about K_d is obtained. In this study, due to the high absorption of oxyphenylbutazone at 295 nm, we have been unable to work at high protein concentration and therefore no attempt was made to determine the concentration of binding sites.

Table 1 summarizes the titration results, expressed as $1/K_d = K_a$, in buffer and in micellar solutions made up with the different surfactants used in this study. Despite the large differences in the quenching efficiency, the association constant K_a remains within the same order of magnitude ($10^5\ M^{-1}$) whether the binding is carried out in buffer or in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate or hexadecyltrimethylammonium bromide. The fraction of quenching rises with the protein concentration, but never exceeds 0.3, as if only a fraction of the albumin molecules present in reverse micelles had retained their oxyphenylbutazone binding capacity.

We have checked that oxyphenylbutazone was indeed solubilized within the aqueous core of reverse micelles and not in isooctane, the organic solvent. Partition experiments were done between isooctane/buffer and isooctane/sodium bis(2-ethylhexyl)sulfosuccinate/buffer. After 15 h of slow shaking, 95% of the oxyphenylbutazone was found either in the aqueous phase or in the reverse micelles. Furthermore, the spectral profile of aqueous solutions of oxyphenylbutazone was identical to that measured in reverse micelles and quite different from the spectrum of oxyphenylbutazone dissolved in isooctane (not shown). In contrast, partition experiments carried out with phenylbutazone (instead of oxyphenylbutazone) revealed that this ligand was completely transferred into isooctane.

Dansylsarcosine-HSA complex in reverse micelles

In another set of experiments, a dansylsarcosine-albumin complex was prepared, as described in Materials and Methods, and the same amount was injected into both 0.1 M borate pH 8.0 and into 0.1 M sodium bis(2-ethylhexyl)sulfosuccinate and hexadecyltrimethylammonium bromide micellar solutions at a W_o value of 20. Fluorescence emission spectra of free and bound dansylsarcosine are shown in Fig. 3. Free dansylsarcosine, whether in aqueous or in micellar solutions, displays a low-intensity spectrum with an ill-defined emission maximum around 570 nm. Once bound to albumin, the fluorescence intensity increases by a factor of almost 70 and the emission maximum is shifted to the blue at 485 nm, as previously reported (Sudlow et al., 1975). After injection of the dansylsarcosine-albumin complex into micellar solutions (anionic or cationic), the intensity of the spectrum decreases dramatically with a simultaneous return of the emission

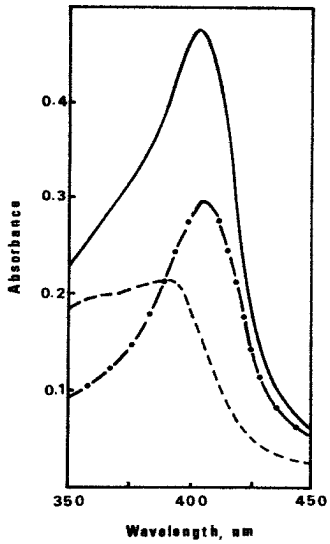

Fig. 4. *Absorption spectra of hemin (— — —), hemin -HSA complex (——) in 0.1 M borate pH 8 and hemin-albumin complex (●——●) in reverse micelles of 0.1 M sodium bis(2-ethylhexyl)sulfosuccinate* $W_o = 11.2$. The concentration of heme is 3.6 μM for all three spectra

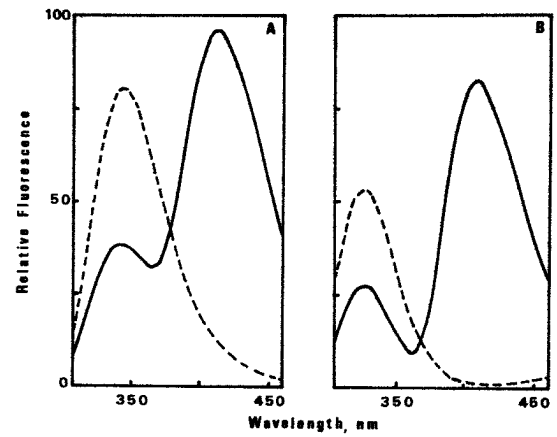

Fig. 5. *Fluorescence emission spectra of HSA (— — —) and anthraniloyl-HSA (——) both at 1.7 μM.* Excitation wavelength was 295 nm. Fluorescence measured in arbitrary units. (A) In 0.1 M phosphate pH 7.8. (B) In reverse micelles of 0.1 M sodium bis(2-ethylhexyl)sulfosuccinate, $W_o = 22.4$

maximum to values higher than 556 nm. This result suggests dissociation of the complex upon micellar incorporation.

To verify this finding, we have measured the partition coefficient of dansylsarcosine between buffer and isooctane as described in the previous paragraph. The results obtained overnight at 20°C indicate that at least 83% of the ligand is present in the aqueous solution. The dissociation is not, therefore, related to preferential solubility of the ligand in the organic solvent. Furthermore, fluorescence polarization experiments demonstrate that dansylsarcosine is released from albumin in the latter medium. Indeed, polarization values of 0.016 and 0.020 were measured for free dansylsarcosine in aqueous and micellar solutions, indicating a free rotation of the fluorophore, while values of 0.27 and 0.010 were measured for the dansylsarcosine-albumin complex in buffer and reverse micelles respectively, also indicating that in the latter medium the ligand has dissociated from albumin.

Hemin-HSA complex in reverse micelles

We have also studied the behaviour of another albumin-ligand complex in reverse micelles. Hemin was selected for its high association constant ($K_a = 1.0 \times 10^8\ M^{-1}$) (Peters, 1985) and for its characteristic spectral properties upon binding in aqueous and in micellar solutions. The absorption spectra of free and albumin-bound hemin are shown in Fig. 4. Free hemin displays an absorption optimum at 385 nm ($\varepsilon = 5 \times 10^4\ M^{-1}\ cm^{-1}$), while upon binding to HSA in aqueous buffer the maximum is shifted to 408 nm, and the molar absorption coefficient increases to $14 \times 10^4\ M^{-1}\ cm^{-1}$ (Moehring et al., 1983). In reverse micelles, the picture is very much the same: free and albumin-bound hemin display spectral patterns similar to those in buffer, but the molar absorption coefficients are lower: $3.5 \times 10^4\ M^{-1}\ cm^{-1}$ for free hemin, increasing after binding to $8.6 \times 10^4\ M^{-1}\ cm^{-1}$ in a similar proportion compared to aqueous solutions. The spectral shift and the substantial increase of the absorption coefficient indicate that, in contrast to dansylsarcosine, most of the hemin ligand remains bound to the protein upon micellar incorporation.

Chemical modifications of HSA

p-*Nitrophenyl anthranilate labelling of albumin.* We have treated the very reactive tyrosine residue (Tyr411) of albumin with *p*-nitrophenyl anthranilate. As previously reported by Hagag et al. (1983), the fluorescence energy transfer between the two aromatic residues (Trp214 and Tyr411) results in tryptophan fluorescence emission quenching observed at 340–350 nm. As illustrated in Fig. 5, in 0.1 M phosphate pH 7.8, the tryptophan is quenched by about 52% in good agreement with the reported results. Upon micellar incorporation, in which the tryptophan emission intensity is already lower by 30% as mentioned above, quenching as a result of *p*-nitrophenyl anthranilate reaction is somewhat less but is still 42%. This indicates that the distance between the two fluorophores is not substantially modified in the micellar system. The peak positions are also changed, blue-shifting upon incorporation into reverse micelles.

Accessibility of Trp214 to oxidation. The oxidation of the sole HSA tryptophan residue by *N*-bromosuccinimide, measured by the decrease of the fluorescence emission, is depicted in Fig. 6. The intensity of the fluorescence maximum is plotted versus the [*N*-bromosuccinimide]/[HSA] molar ratio in buffer and in micellar solutions at $W_o = 22.4$. As observed previously, the initial fluorescence level is lower by 30% in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate and hexadecyltrimethylammonium bromide compared to the value obtained in buffer. However, while in 0.1 M acetate pH 4.4 and in the cationic micellar solution the curves are biphasic, in the anionic surfactant the curve has a single slope over all but the last few points. It has also to be emphasized that the amount of *N*-bromosuccinimide consumed in the reaction is higher in micellar solutions (17 mol *N*-bromosuccinimide/mol HSA), than in buffer (13 mol/mol; Spande and Witkop, 1967). We have checked that the *N*-bromosuccinimide consumed in the reaction was used for tryptophan and not tyrosine oxidation by recording the protein fluorescence emission spectrum upon excitation at 280 nm before and after the reaction. In hexadecyltrimethylammonium bromide micellar solutions,

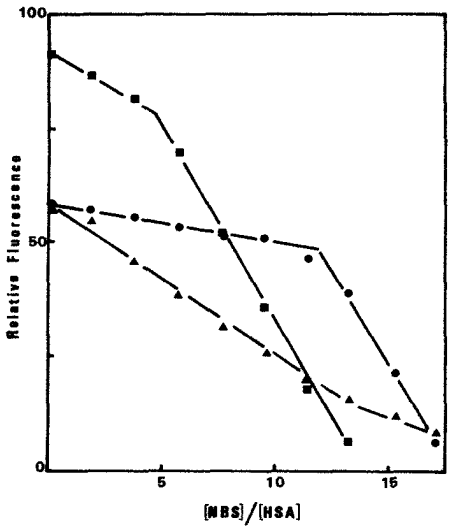

Fig. 6. *Oxidation of Trp214 by* N-*bromosuccinimide in 0.1 M acetate pH 4.4 (■) and in 0.1 M sodium bis(2-ethylhexyl)sulfosuccinate (●) and hexadecyltrimethylammonium bromide (▲) reverse micelles,* $W_o = 22.4$ *in the same buffer.* 1 ml 4.8 μM albumin solution was titrated with successive additions of 0.92 mM *N*-bromosuccinimide (NBS) in buffer and in reverse micelles. The excitation wavelength was 295 nm

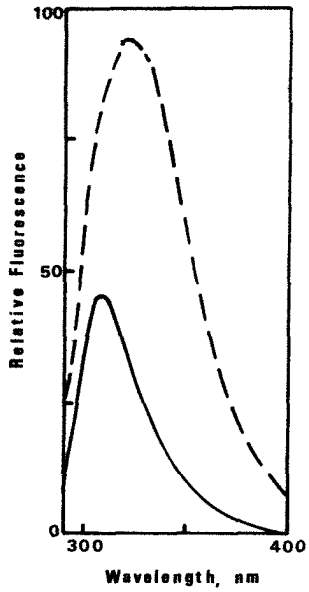

Fig. 7. *Fluorescence emission spectra of albumin before (– – –) and after (——) oxidation by* N-*bromosuccinimide in hexadecyltrimethylammonium bromide reverse micelles.* Experimental conditions as in Fig. 6. Excitation at 280 nm

the maximum emission of the unreacted protein is located at 321 nm, while after oxidation the overall intensity is reduced by 50% and the maximum is located at 307 nm indicating that the remaining fluorescence is mainly generated by the phenolic side-chains. In sodium bis(2-ethylhexyl)sulfosuccinate reverse micelles, the values are more blue-shifted; to 310 nm and 304 nm for native and oxidized HSA, respectively (Fig. 7).

Circular dichroism

Incorporation into reverse micelles leads to a loss of intensity in both the near and far ultraviolet regions of the spectrum. In the far ultraviolet the ellipticity in the micelles is 20–25% less than in aqueous buffer (Fig. 8). Secondary structure calculations by the method of Chang et al. (1987) indicate a loss of aproximately 15% of the α-helical content in agreement with the recent work of Marzola et al. (1990). The spectrum in the near ultraviolet shows negative ellipticity at all wavelengths, becoming more negative above 290 nm upon incorporation into micelles and less negative below that wavelength. The changes in the area of 295–300 nm (data not shown) are consistent with involvement of the single tryptophan at position 214, although some involvement of the disulfides in this spectral region cannot be ruled out (Kahn, 1979). Below 290 nm, effects on the 18 tyrosine residues are seen. Although the magnitude of the change in ellipticity per tyrosine is smaller than for the tryptophan, 1.7 deg cm^2/dmol versus 5 deg cm^2/dmol (mean residue basis), we cannot distinguish between a large effect on one or a few tyrosines from smaller effects distributed over most or all of them.

Hydrodynamic radius, R_H*, of HSA-containing reverse micelles*

The results of the measurements of D_S, the self-diffusion coefficient, by FRAPP are illustrated in Fig. 9. In this type of experiment only FTC-albumin-containing micelles display

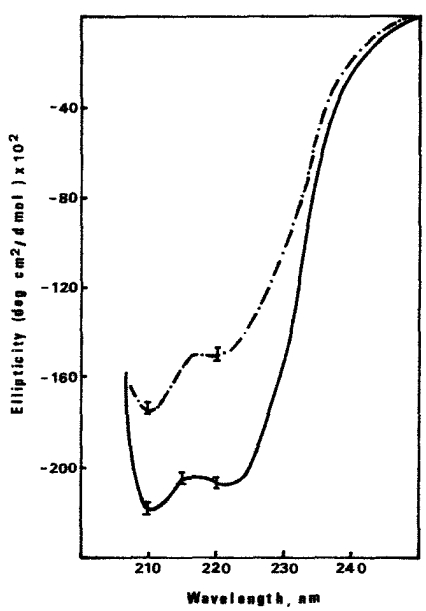

Fig. 8. *The far ultraviolet CD spectrum of HSA in 0.05 M Tris pH 8.0 (——) and in reverse micelles, same buffer,* $W_o = 22.4$ *(●——●).* the protein concentration was 17.5 μM and 16.6 μM, respectively, the cell path was 0.1 cm. Results are expressed on a mean residue basis

fluorescence and contribute to R_H. The apparent hydrodynamic radius is calculated from the Stokes-Einstein relation, $kT/6\pi\eta D_S$, where k is the Boltzmann constant, T the absolute temperature and η the isooctane viscosity (491 Pa · s). The plot of R_H versus the sodium bis(2-ethylhexyl)sulfosuccinate concentration extrapolates to $R_H = 7.3$ nm for zero sodium bis(2-ethylhexyl)sulfosuccinate concentration. The HSA molecule, depicted as an oblate ellipsoid with axes of 14 nm by

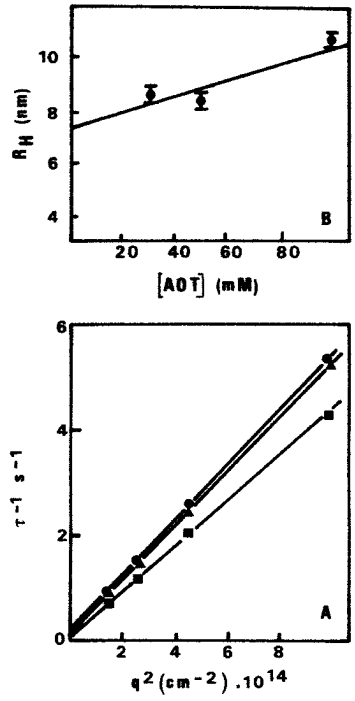

Fig. 9. *Determination of the FTC-albumin hydrodynamic radius* R_H *in sodium bis(2-ethylhexyl)sulfosuccinate reverse micelles at* W_o = *22.4, 0.03 M Tris pH = 8.6 by photobleaching experiments.* (A) Plot of the reciprocal recovery time τ^{-1} versus $q^2 = (2\pi i)^2$ (see text) at three different concentrations of sodium bis(2-ethylhexyl)sulfosuccinate (AOT): 0.03 M (●), 0.05 M (▲) and 0.1 M (■). (B) Plot of R_H apparent versus [sodium bis(2-ethylhexyl)sulfosuccinate]. The R_H value extrapolates to 7.3 nm, for zero [sodium bis(2-ethylhexyl)sulfosuccinate]

4 nm (Peters, 1985), can therefore be accomodated within an equivalent sphere of 7.5 nm radius. Empty micelles at W_o = 22.4 have a hydrodynamic radius of 5 nm. Upon addition of the protein, the solution reorganizes (Chatenay et al., 1985) to yield HSA-containing micelles of 7.3 nm radius as well as empty ones. Thus a protein-containing micelle can hardly host more than a single albumin molecule.

DISCUSSION

Biological processes occurring at membranes can be studied at different levels. At a low degree of complexity, reverse micelles constitute a good membrane-mimetic system (Nicot et al., 1985; Leodidis and Hatton, 1990) for the study of the complex physicochemical forces acting at interfaces of multicomponent assemblies (Israelachvili and McGuiggan, 1988). The large size (66.5 kDa) and the number of amino acids (585) in HSA provide the opportunity for multiple protein-micellar surface interactions which would contribute to the process of conformational changes described herein.

Conformational alterations

The α helix loss (15%) taking place upon micellar incorporation is in good agreement with the preliminary CD observations of Marzola et al. (1991) who have also reported structural changes of HSA measured by ESR spectroscopy, under very similar experimental conditions. In contrast, the ellipticity of HSA measured in aqueous solutions, at several sodium bis(2-ethylhexyl)sulfosuccinate concentrations (under critical micellar concentration), remains unaffected (P. Marzola, personal communication). Thus, the changes observed in micellar solutions cannot be explained by the binding of surfactant monomers to the protein, followed by unfolding and denaturation. They are rather due to the complex interactions of a protein, displaying a well documented conformational flexibility (Weber, 1975) with the micellar interface.

In addition, our results provide information about local structural perturbations. The blue shift of the fluorescence emission maximum (excited at 295 nm) from 344 nm in buffer to 327 nm in sodium bis(2-ethylhexyl)sulfosuccinate reverse micelles indicates a much less polar environment of Trp214 in the latter medium. This effect does not seem to be closely related to micelle–protein electrostatic interactions, since in hexadecyltrimethylammonium bromide, a surfactant with charge complementary to that of HSA, the maximum emission is shifted to 330 nm. Furthermore, the blue shift is not due to the binding of surfactant monomers to the protein, since the maximum emission value is unaffected by the surfactant concentration. Finally, the fluorophore is also shielded from the solvent of the aqueous micellar water pool since the maximum is also independent of the water content of the system in the $W_o = 12-30$ range, suggesting a deeper burial of the indole into the hydrophobic fold (Eftink and Ghiron, 1977).

The perturbation of the sole tryptophan microenvironment in micellar solutions is also evidenced by the *N*-bromosuccinimide titration curves. The results of the oxidation experiments indicate that the accessibility of Trp214 in reverse micelles is probably hindered by the nature of the interactions between the polypeptide chain and the micellar wall. This is suggested by the increase of the consumption of *N*-bromosuccinimide/albumin (from 12–13 to 17–18 mol/mol). From kinetic experiments carried out with HSA, Peterman and Laidler (1980) concluded that the reaction could be described by a rather complex process, which precludes any precise interpretation of the titration curves at the molecular level (Fig. 6). Recent time-resolved fluorescence measurements of albumin (Gentin et al., 1990) suggest a high internal flexibility in the Trp vicinity which could become 'frozen' in reverse micelles (Waks and Beychok, 1974). Whatever the detailed explanation, it can be concluded that the environment in the vicinity of Trp214 is altered in reverse micelles.

Hagag et al. (1983) measured, by resonance energy transfer the distance between Trp214 and Tyr411, a particularly reactive phenolic side-chain of HSA. It is clear from comparison of Fig. 5A and B that we are dealing with the same process in buffer and in reverse micelles. In the latter medium, the fluorescence emission of anthraniloyl-HSA (excited at 295 nm) is only quenched 10% less when compared to the same phenomenon in buffer. It would therefore appear that the distance between the two fluorophores (2.5 nm), located in different domains of the albumin molecule, is not markedly affected by the incorporation of the protein into reverse micelles and by the resulting conformational changes.

Ligand binding in reverse micelles

These studies are hampered by experimental difficulties. The ideal ligand should (a) solubilize in the aqueous core of reverse micelles (water pool) rather than in the surrounding organic phase and (b) display a reasonably good association constant (in the 10^6-10^5 M^{-1} range). Furthermore the spectroscopic characteristics of the free and bound ligands should

differ significantly in aqueous and micellar solutions. In our hands, very few ligands among those currently used in albumin-binding studies meet all the above requirements.

During the course of this investigation we had to deal with three different ligand-binding situations. The most dramatic effect observed concerns the dansylsarcosine-HSA complex. Since we were unable to detect any measurable binding by increase of fluorescence intensity at 485 nm, we have incorporated a previously prepared complex into reverse micelles. It is evident from Fig. 3 that the complex dissociates in micellar solution. This result is confirmed by static fluorescence polarization measurements and by the value of the partition coefficient, indicating that dansylsarcosine has no spontaneous tendency to partition into the organic solvent. In contrast, the hemin-HSA complex does not seem affected by similar experimental conditions, as illustrated by the maximum and the high value of the absorption coefficient in the Soret region. The binding may thus stabilize the conformation assumed by the protein. We may now consider the intermediate case, the binding of oxyphenylbutazone. The binding constants measured by absorption spectroscopy or fluorescence emission quenching are of the same order of magnitude whether in buffer or in reverse micelles ($K_a = 10^5\ M^{-1}$). The main difference resides in the quenching level at saturating values of oxyphenylbutazone concentration, indicating that only a fraction of the albumin molecules has retained the binding properties. How can these differences be explained?

A working hypothesis, which we favor, is that the association constants of the three ligands are lowered, for example, by several orders of magnitude when going from the aqueous to the micellar solution. This is due to the interactions of the albumin molecule with the large surface generated by reverse micelles and to the subsequent changes in conformation observed. At the protein concentration used in this work, hemin would still remain bound to HSA, while dansylsarcosine and a substantial fraction of oxyphenylbutazone would dissociate to a large extent. The fact that the association constants for the complexes formed by HSA and dansylsarcosine ($K_a = 1.8 \times 10^5\ M^{-1}$; Sudlow et al., 1975) or oxyphenylbutazone ($K_a = 1.6 \times 10^5\ M^{-1}$; Elbary et al.,1982) have identical values in buffer does not imply that they decrease by the same amount upon micellar incorporation.

We know that the chemical nature of the three ligands used in this study is quite different and it is therefore reasonable to assume a different binding mechanism for each of them. In contrast, we are not in a position to localize precisely the three binding sites on the albumin primary sequence, although we know they do not belong to the same domains of the albumin molecule (Peters, 1985). They may therefore react differently to micellar incorporation and their association constant might be affected to a different extent by the change in albumin conformation. Of course, albumin polymerization induced by micelar incorporation could possibly either prevent the binding of ligands or favor the dissociation of the ligand-HSA complex. This mechanism can, however, be ruled out since the results of FRAPP measurements are consistent with the presence of but a single albumin molecule per micelle.

The sequence of events could be summarized as follows: upon micellar incorporation albumin undergoes a surface-induced conformational change also observed by Horie et al. (1988) by ESR spectroscopy upon contact of HSA with hepatocytes. The surface interaction with HSA would give rise to the two albumin subpopulations described by Reed and Burrington (1989). The first would have an enhanced affinity for the inner micellar surface, a 'flattened' surface conformation, and a lowered affinity for the ligand. The second would display a low surface affinity and an unchanged binding constant compared to plain buffer, depending on the ligand. This scheme would indeed provide a facilitating mechanism for ligand release occurring upon cellular membrane contact (Forker and Luxon, 1983; Fleischer et al., 1986).

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