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CATALYSIS BY HEMOPROTEINS AND THEIR STRUCTURAL ORGANIZATION IN REVERSED MICELLES OF SURFACTANTS IN OCTANE

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The cytochromes *P*-450 LM-2 and b_5 from rabbit liver microsomes have been entrapped into reversed micelles of surfactants in octane. The optimum conditions providing for the maximum stability of the hemoproteins have been found: pH and concentration of the buffer solution, the glycerol addition, the surfactant concentration, the $[H_2O]/[surfactant]$ ratio and, finally, the reversed micelles composition including aerosol OT and its mixture with Triton X-45, Tween 20 and cetyltrimethylammonium bromide (CTAB). The transformation kinetics of the hemoproteins solubilized by detergents has been studied by monitoring the absorbance of hemoproteins in the Soret band region. Significant changes in tryptophan fluorescence of cytochrome b_5 and in CD spectra of myoglobin in reversed micelles and their dependence on the $[H_2O]/[aerosol\ OT]$ ratio have been shown. The three hemoproteins in reversed micelles have been found to exhibit high catalytic activity with respect to their reaction with cumene hydroperoxide. The kinetic and spectral data reveal the structural transformations of the proteins entrapped into the micelles due to the interactions of the lumenal surface of the micelles and the protein molecule surface.

Introduction

Recently, a new mode of studying protein functions has been introduced: it has been proposed that enzymes be dissolved in a colloid solution of water in an organic solvent rather than in water [1–10]. In such systems, many enzymes, regardless of their nature and function, retain catalytic activity, e.g., chymotrypsin, trypsin, lysozyme, ribonuclease, pyrophosphatase, peroxidase, alcohol dehydrogenase, pyruvate kinase, catalase and some forms of cytochrome *P*-450 [1–10]. It is important to study the interaction of the reversed micelles with the proteins solubilized by these micelles. A comprehensive study of the system 'protein-aerosol OT-water-octane' by sedimentation analysis has shown that the protein molecule is introduced into the lumen of the reversed micelle with a stoichiometric ratio of 1 : 1 independently of the outer size of the reversed micelles [11].

The effect of the reversed micelles on the solubilized protein has not been thoroughly investigated, although it is of great importance for two reasons: first, it is indispensible for elucidating the type of change in the protein structure in the reversed micelles as compared to that in aqueous solutions and in a crystal state; second, the reversed micelles of surfactants with entrapped proteins are a realistic model of biological membranes [12,13]; the study of their mutual effects may throw light on the ultrastructure of biomembranes.

It is most convenient to study the effect of

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Abbreviations: CTAB, cetyltrimethylammonium bromide; aerosol OT, the sodium salt of di(2-ethylhexyl)sulfosuccinic acid.

reversed micelles on the structural organization of the proteins enclosed therein using hemoproteins whose natural label, heme, facilitates control of the protein state, since spectral characteristics of hemoproteins (maxima and intensity of the Soret bands, fluorescence and CD spectra) reflect changes occurring in proteins. At the beginning of our experiments, cytochrome *c*, catalase and peroxidase [2,3,6], cytochrome *P*-450$_{cam}$ from *Pseudomonas putida* that hydroxylates camphor [6], cytochrome *P*-450 LM-2 and cytochrome b_5 from rabbit liver microsomes [8,10] and myoglobin [14] were entrapped into reversed micelles in octane. Changes of maximum positions and intensities of the Soret bands revealed drastic changes occurring in the hemoproteins [6,8–10].

The aim of the present work was a systematic study of structural changes occurring in three hemoproteins, cytochromes *P*-450 LM-2 and b_5 and myoglobin, in the reversed micelles of surfactants in octane. To achieve this, the following spectral methods have been employed: (i) electron spectroscopy to monitor the Soret band intensities, (ii) tryptophan fluorescence and (iii) circular dichroism spectra. The processes of hemoprotein transformation have been studied kinetically. For cytochrome b_5 the energy characteristics of transformation in the reversed micelles of aerosol OT have been obtained. The spectral and kinetic data gave evidence of structural changes occurring in protein molecules in micelles of surfactants, which in turn affect functional characteristics of the solubilized hemoproteins. The data obtained permit us to propose a model for the system 'protein-surfactant-water-octane'.

Experimental

Materials

Cytochrome *P*-450 LM-2 and cytochrome b_5 were isolated from rabbit liver microsomes and purified to a homogeneous state as previously described [15,16]. Myoglobin from whale heart (Serva, Heidelberg, F.R.G.) was used without further purification. To obtain the reversed micelles of surfactants in octane, the sodium salt of di(2-ethylhexyl)sulfosuccinic acid (aerosol OT) (Merck, F.R.G., or Fluka, Switzerland), Triton X-45 (Sigma, U.S.A.), Tween 20 (Merck) and cetyltrimethylammonium bromide (Chemapol, Czechoslovakia) have been used. Octane and cumene hydroperoxide were redistilled before use.

Preparation of the reversed micelles of surfactants in octane

The preparation of reversed micelles in octane and the inserting of the hemoproteins was performed using the technique of Balny and Douzou [6] with our modifications [8]: a saturated solution of CTAB and Tween 20 in octane was prepared at room temperature, and Triton X-45 was then added to a concentration of 0.01 M. The solution was kept overnight and then centrifuged at $1000 \times g$ for 10 min. The precipitate was discarded and aerosol OT was dissolved in the supernatant to a concentration of 0.2 M. In the case of single and mixed binary micelles of aerosol OT and Triton X-45 in octane at room temperature, 'dry' solutions of 0.4 M aerosol OT and 0.3 M Triton X-45 in hydrocarbon were prepared. These solutions were employed as the starting solutions in the preparation of reversed micelles. To a 'dry' solution of surfactants in octane, the proteins in a potassium phosphate buffer or in 0.05 M Tris/H_3PO_4 buffer containing 15% glycerol were added (water phase concentration was 1–12% v/v). Then the system was ultrasonically dispersed. As a result, optically transparent systems permitting spectral and kinetic measurements were prepared.

Spectral methods

The protein absorption spectra in the reversed micelles were recorded in thermostatically controlled cuvettes using a Specord UV VIS spectrophotometer (Carl Zeiss, Jena, G.D.R.) at room temperature (20°C) and at 31°C. The fluorescence spectra were recorded with an SLM-4800 spectrofluorimeter (U.S.A.) in 1-cm cuvettes. The samples contained 0.05 M Tris/H_3PO_4 buffer, pH 7.5, with 15% glycerol and either oxidized cytochrome b_5 or reversed micelles of surfactants (0.25 M) in octane (1–10% water phase) with solubilized hemoprotein in the same buffer. After addition of the protein into a 'dry' surfactant solution, the sample was shaken for 1 min and after 3–4 min the fluorescence spectra were recorded at room temperature (20°C).

The CD spectra were recorded at room temper-

ature with a Jasco J-20 spectropolarimeter (Japan) in 0.2-cm cuvettes (ultraviolet region) and in 1-cm cuvettes (visible region). The samples contained myoglobin in 0.05 M Tris/H_3PO_4 buffer, pH 7.5, with 15% glycerol or the reversed micelles of aerosol OT (0.25 M) with solubilized hemoprotein dissolved in the same buffer (1–20%). After the addition of hemoprotein to the 'dry' aerosol OT solution in octane, the sample was stirred for 1–2 min and after 2–3 min the CD spectra were recorded.

Kinetic measurements

Hemoprotein transformations in the reversed micelles were followed by changes in the Soret band intensity at 31°C. The rate constants of hemoprotein transformation, k in s^{-1}, were calculated from the dependences of $\log(\Delta A/\Delta A_0)$ on time, where ΔA_0 and ΔA designate the hemoprotein absorption intensity at the starting point and after a certain time period, respectively.

The reactions of cumene hydroperoxide with hemoproteins were carried out with a great excess of cumene hydroperoxide and characterized by the rate constants of protein destruction, k in s^{-1}, calculated from the $\log(\Delta A/\Delta A_0)$ dependencies on time.

Results and Discussion

Optimal conditions for hemoprotein solubilization by reversed micelles of surfactants

In Fig. 1 the absorption spectra of cytochromes *P*-450 LM-2 and b_5 and myoglobin in the reversed micelles of surfactants and in buffer solution are shown. It is seen that the entrapping of hemoprotein into the micelles is accompanied by a shift in the Soret band maximum which is slight for myoglobin and cytochrome b_5 and pronounced for cytochrome *P*-450 LM-2. The magnitude of the Soret maximum shift depends on the composition of micelles (see Table I). The Soret band shift coincides with the moment of hemoprotein entrapping into the micelles. The intensity drop of the Soret band is prolonged, and the rate of this process is determined by several factors to be discussed below.

1. Protein concentration. The stability of cytochrome b_5 in the aerosol OT micelles increases with protein concentration up to 4 μM, after which it remains practically unchanged. At a higher cytochrome b_5 concentration (6.78 μM) its transformation is characterized by a single phase, whereas at low concentration (0.68 μM) two phases, a slow and a fast one, of protein transformation are observed.

TABLE I

THE RATE CONSTANTS OF CYTOCHROME *P*-450 LM-2 TRANSFORMATION IN REVERSED MICELLES OF SURFACTANTS IN OCTANE AT 30°C

4.95% 0.05 M phosphate buffer, pH 6.6.

Composition of the micelles	$k \times 10^5$ (s^{-1})		Absorption λ_{max}(nm)
	First phase	Second phase	
1 Aerosol OT	9.1	2.2	406
2 Aerosol OT + Triton X-45	5.3	2.2	403
3 Aerosol OT + Tween 20	8.7	1.8	403–407
4 Aerosol OT + CTAB	9.1	1.5	411
4 Aerosol OT + CTAB + Triton X-45	2.2	0.6	402
6 Aerosol OT + CTAB + Tween 20	2.7	0.3	403–406
7 Aerosol OT + CTAB + Tween 20 + Triton X-45	1.4	—	402–404

2. The [H_2O] / [surfactant] ratio. The highest stability of cytochromes *P*-450 LM-2 and b_5 in micelles is observed at a concentration of aerosol OT of approx. 0.25 M. The use of mixed micelles composed of aerosol OT and Triton X-45 (1 : 2 ratio) does not alter the optimal total concentration of the surfactants. In Fig. 2 the effect of the parameter n ($n = [H_2O]/[\text{surfactant}]$) on the rate constant of cytochrome b_5 transformation at 31°C is shown. In these experiments the concentrations of aerosol OT and protein were 0.25 M and 4.34 μM, respectively. At $n \simeq 10$ the highest stability of cytochrome b_5 in micelles was achieved, the Soret band intensity and the maximum absorption being not essentially different from those in solution. A deviation from the optimum n value to either side resulted in a small blue shift of the Soret band maximum (3–6 nm) and a decrease in its intensity. In Fig. 2 is shown the effect of buffer concentration in the reversed micelles on the rate constants of cytochrome *P*-450 transformation: these decrease in inverse proportion to the water content

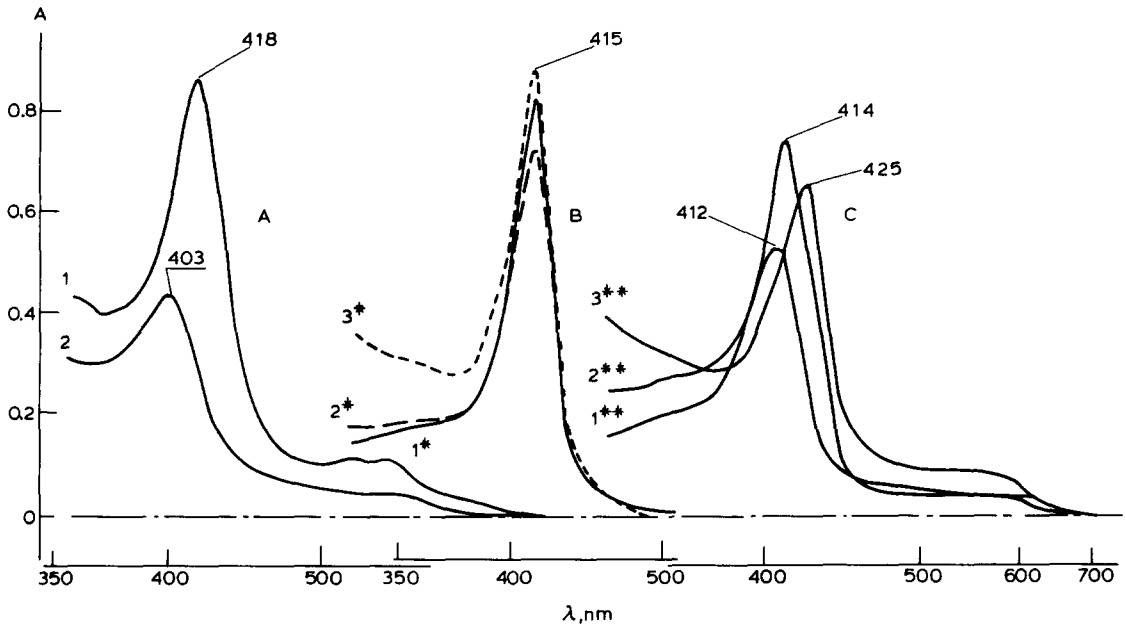

Fig. 1. Absorption spectra of hemoproteins in buffer solution and in reversed micelles of surfactants in octane. A, cytochrome *P*-450 LM-2: 1, phosphate buffer, pH 7.25; 2, the mixed reversed micelles containing the same buffer (5.2% vol.). B, cytochrome b_5: 1*, Tris/H_3PO_4 buffer, pH 7.5, 15% glycerol; 2*, the aerosol OT micelles (4.2% polar phase); 3*, the mixed reversed micelles of aerosol OT : Triton X-45 (1 : 5) with polar phase content of 4.2%. C, myoglobin: 1**, Tris/H_3PO_4 buffer, pH 7.5, with 15% glycerol; 2**, the aerosol OT micelles (4.2% polar phase), and 3**, the mixed micelles of aerosol OT-Triton X-45 (1 : 5) with the polar phase content of 4.2%.

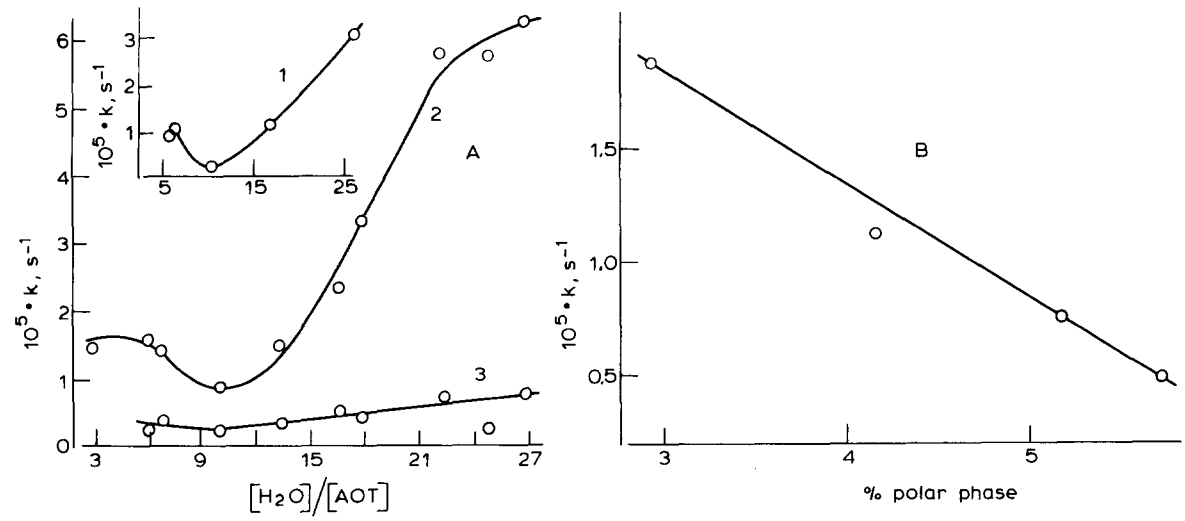

Fig. 2. A, effect of the $[H_2O]/[$aerosol OT$]$ (AOT) ratio on the rate constants of the cytochrome b_5 transformation (31°C, 0.25 M aerosol OT, 4.34 μM cytochrome b_5): 1, first; 2, second, and 3, third phase of the process. B, the dependence of the rate constant of the cytochrome *P*-450 transformation on 0.15 M phosphate buffer content in the mixed reversed micelles (system 7, see Table I) in octane at 30.5°C.

as it increases from 3 to 6%, i.e., the stability of cytochrome *P*-450 increases with micelle wetness.

3. Composition of reversed micelles. In reversed micelles of different compositions two phases of cytochrome *P*-450 transformation are observed, with a shift of the Soret band maximum, the magnitude of which depends on the composition of micelles. A more complex micelle composition leads to increased stability of cytochrome *P*-450. Maximum stability of the protein is observed in system 7, where the micelles consist of four components (Table I). In contrast with cytochrome *P*-450, cytochrome b_5 is most stable in either single aerosol OT micelles or mixed micelles with a maximum content of Triton X-45. At an equimolar ratio of the two surfactants the stability of cytochrome b_5 is lowest (Fig. 3).

4. pH of the buffer solution. According to our data [17], cytochrome *P*-450 in the liver microsomes is most stable at pH 6.6. It is interesting, therefore, to compare the effect of pH on the stability of cytochrome *P*-450 in microsomes and in reversed micelles of surfactants. In the mixed-micelle system 7 (see Table I), over the pH region from 6.6 to 7.8 the rate of cytochrome *P*-450 transformation in the first phase was unchanging (Fig. 4). The character of the dependence for the second phase of protein transformation was somewhat different: k was minimal at pH 7.5.

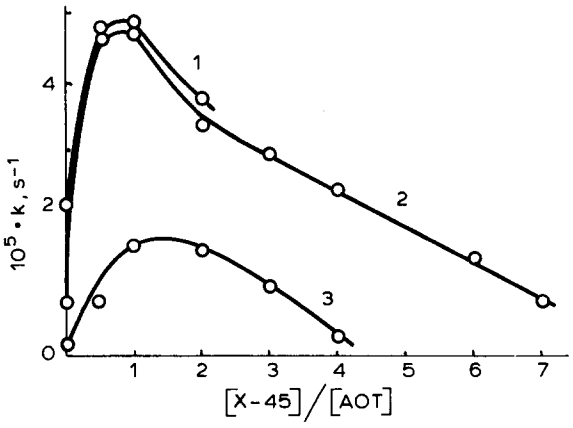

Fig. 3. Effect of the Triton X-45 : aerosol OT (AOT) ratio in the reversed micelles on the rate constants of the cytochrome b_5 transformation (31°C, 0.25 M surfactants, 4.5% polar phase): 1, first; 2, second, and 3, third phase of the process.

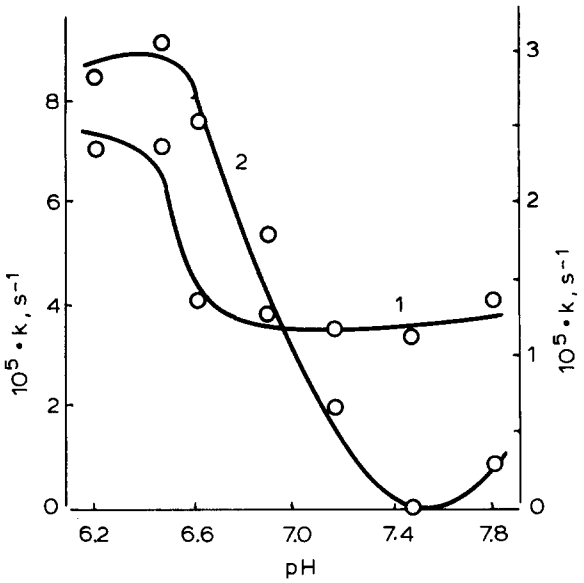

Fig. 4. Effect of pH of 0.05 M phosphate buffer on the rate constants of the cytochrome *P*-450 transformation in the mixed reversed micelles (aerosol OT + CTAB + Tween 20 + Triton X-45) at 30.5°C: 1, first; 2, second phase.

5. Concentration of buffer and presence of glycerol in the mixed reversed micelles. An increase in the concentration of potassium phosphate buffer from 0.01 to 0.3 M causes a 12-fold increase in stability of cytochrome *P*-450 in the reversed mixed micelles. When the buffer concentration increases above 0.2 M, the character of cytochrome *P*-450 transformation is changed: the kinetic curves are not described by the first-order equation. It is well known that the presence of glycerol and other polyols stabilizes cytochrome *P*-450 against denaturation [17,18]. Glycerol stabilizes the reversed micelles as well [19]. The addition of 15% glycerol leads to maximum stabilization of cytochrome *P*-450 in the mixed reversed micelles of surfactants [8].

6. Temperature. The temperature dependence of the rate constants of cytochrome b_5 transformation in a buffer solution in the presence of glycerol during the first phase of the process is characterized by activation energy values of 4–5 kcal/mol; for the reversed aerosol OT micelles the activation energy is 2 kcal/mol, and for the mixed micelles of aerosol OT : Triton X-45 (1 : 1) $E = 6.6$ kcal/mol. The subsequent transformation phases

either depend slightly or do not depend at all on temperature. Identical spectral changes occur for the three systems with a temperature increase: the Soret band broadens, its maximum drops and is shifted by several nanometers to the shorter-wavelength region. In the buffer solution, the maximum shifts from 415 to 408 nm; in micelles, from 414 to 409 nm, and in binary micelles of aerosol OT and Triton X-45, from 415 to 407 nm.

Thus, the optimal conditions for hemoprotein solubilization in the reversed micelles of surfactants are determined by the protein concentration, composition of micelles and the $[H_2O]/[surfactant]$ ratio, pH of buffer solution, temperature of the system and presence of the stabilizing compounds (glycerol, polyols), the buffer concentration and, finally, by the nature of the protein. Our data indicate that the integral membrane protein, cytochrome *P*-450 LM-2, is most stable in mixed micelles, whereas the other membrane protein, cytochrome b_5, is most stable in single reversed micelles. Entrapping of hemoproteins into the reversed micelles is followed by protein changes leading to a decrease in the Soret band intensity and to a shift of its maximum. What is the cause of such alterations? To answer this question we have studied the fluorescence spectra of myoglobin and cytochrome b_5 and CD spectra of myoglobin in the reversed micelles of aerosol OT in octane.

Alterations in fluorescence and CD spectra of hemoproteins entrapped into the reversed aerosol OT micelles

In Fig. 5 are presented the fluorescence spectra of the oxidized cytochrome b_5 in a buffer solution (curve 1) and in the reversed aerosol OT micelles in octane (curves 3–5). Upon incorporation of cytochrome b_5 into aerosol OT micelles, a downshift of the fluorescence maximum from 329 to 324 nm occurs together with an increase in fluorescence intensity. Fig. 5 also illustrates the effect of n on the intensity of the tryptophan fluorescence of cytochrome b_5 in the aerosol OT reversed micelles. The highest fluorescence intensity is observed at $n = 3–5$, and with an increase in the water content in micelles the fluorescence intensity drops to a practically constant value. With mixed micelles consisting of aerosol OT and Triton X-45 (1 : 1), a fluorescence quenching has been observed

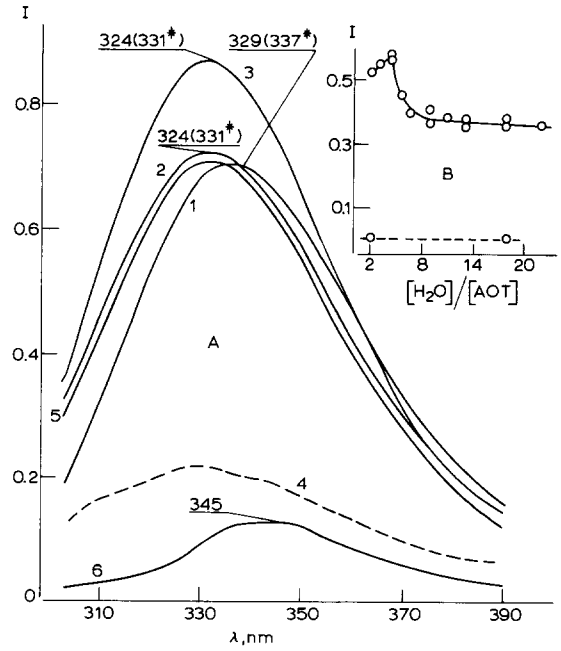

Fig. 5. A, fluorescence spectra of cytochrome b_5 (3.24 mM) at room temperature: 1, hemoprotein in 0.05 M $Tris/H_3PO_4$ buffer, pH 7.5, 15% glycerol; 2, the same system + 0.13 mM octane + 5 mM aerosol OT; 3, cytochrome b_5 in the micelles of aerosol OT (0.25 M) with 2% polar phase; 4, the empty aerosol OT micelles; 5, cytochrome b_5 in the mixed micelles (0.25 M surfactants) with aerosol OT : Triton X-45 ratio of 1 : 1, and mixed empty micelles. B, effect of the $[H_2O]/[$aerosol OT$]$ (AOT) ratio on the fluorescence intensity of cytochrome b_5 (1.62 μM) in the aerosol OT micelles in octane. The dotted line shows the fluorescence level for the empty micelles.

(Fig. 5, curves 1 and 6).

A transition from aqueous solutions to the reversed aerosol OT micelles is characterized by drastic changes in the CD spectrum of myoglobin. The intensity of the far ultraviolet region spectra is determined by the water content in the reversed micelles. Fig. 6 shows dependence of the molar ellipticity in the reversed aerosol OT micelles on the n value. It is seen that molar ellipticity increases monotonically with increasing n, approaching but not reaching the θ values for the aqueous myoglobin solution.

The spectral data obtained may be interpreted in the following way. The fluorescence of cytochrome b_5 caused by tryptophan residues increases in intensity and is characterized by a maximum blue shift after the protein transfer from aqueous

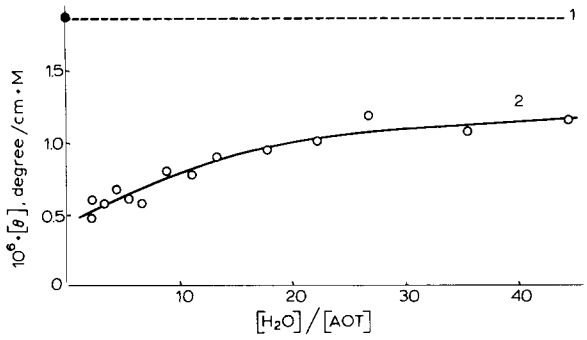

Fig. 6. Effect of the $[H_2O]/[$aerosol OT$]$ (AOT) ratio on the molar ellipticity of myoglobin (220 nm): 33.3 μM hemoprotein, 0.25 M aerosol OT, 1–20% polar phase.

solution to the reversed aerosol OT micelles. This observation is characteristic of proteins with tryptophan in a highly hydrophobic environment. The fluorescence intensity decreases with the water content in micelles, since the water structured by micelles is close to tryptophan residues and causes the fluorescence quenching. Triton X-45 decreases the fluorescence of cytochrome b_5, since its tryptophan is located close to the aromatic fragments of detergent. The tryptophan fluorescence of cytochrome b_5 suggests a strong interaction of hemoprotein and the lumenal surface of the reversed micelles.

CD and X-ray analysis revealed that in aqueous solution and in a crystal state myoglobin contains 77% α-helical structure [20]. During the transfer of myoglobin from aqueous solutions into reversed micelles a decrease of molar ellipticity in the far ultraviolet region that suggests an alteration in the α-helical fraction is observed. An approximate evaluation shows that the content of α-helix in myoglobin decreases 2–5-fold in the reversed micelles at 5% polar phase as compared to aqueous solutions of myoglobin. The increase of polar phase content enhances the α-helix content in the reversed aerosol OT micelles. However, the increase in the water content in micelles does not produce the α-helix level characteristic of aqueous myoglobin solutions (Fig. 6). According to our observations, a change in the absorption intensity in the ultraviolet region of the CD spectra proceeds immediately after the protein entrapping into micelles without any further alterations. Thus, the entrapping of hemoproteins into the reversed micelles of surfactants is accompanied by an essential change in the protein secondary structure.

It is of practical importance to discover whether hemoproteins entrapped into the reversed micelles retain their catalytic functions. To answer this question, we have studied the reaction of cumene hydroperoxide with the three hemoproteins inserted into the reversed aerosol OT micelles in octane.

Interaction of hemoproteins with cumene hydroperoxide in the reversed micelles of surfactants in octane

Upon addition of cumene hydroperoxide into aqueous solutions of hemoproteins or to the solubilized reversed micelles, a rapid complex formation of hydroperoxide with proteins occurs [16,21]. The hemoprotein complexes with cumene hydroperoxide are decomposed with time, accompanied by a decrease in the Soret band intensity. The kinetics of the complex transformation for the three hemoproteins is properly described by the first-order equation from which the rate constants for the complex destruction in s^{-1} may be calculated. The transformation of the cumene hydroperoxide-hemoprotein complexes is generally characterized by one or two phases both in aqueous solutions and in the reversed micelles in octane.

In Fig. 7 the dependencies of the effective rate constants, k, on initial cumene hydroperoxide concentration ($[CHP]_0$) in buffer solution of the three proteins are presented, k being proportional to $[CHP]_0$ for myoglobin (curve 2). For cytochrome *P*-450, this dependence is of a hyperbolic character, and for cytochrome b_5 a sharp increase in the rate constant at $[CHP]_0 > 1.5$ mM has been observed (curve 3). The insertion of proteins into the reversed aerosol OT micelles dramatically alters the character of the dependence of k on $[CHP]_0$ for cytochrome b_5 (Fig. 8, curve 3), whereas for cytochrome *P*-450 and myoglobin the dependencies are not changed as compared to the aqueous solutions of these proteins. Upon insertion of hemoproteins into the mixed reversed micelles composed of aerosol OT and Triton X-45, a decrease of the rate of hemoprotein transformation in the reaction with cumene hydroperoxide has been observed. The dependencies of k on $[CHP]_0$

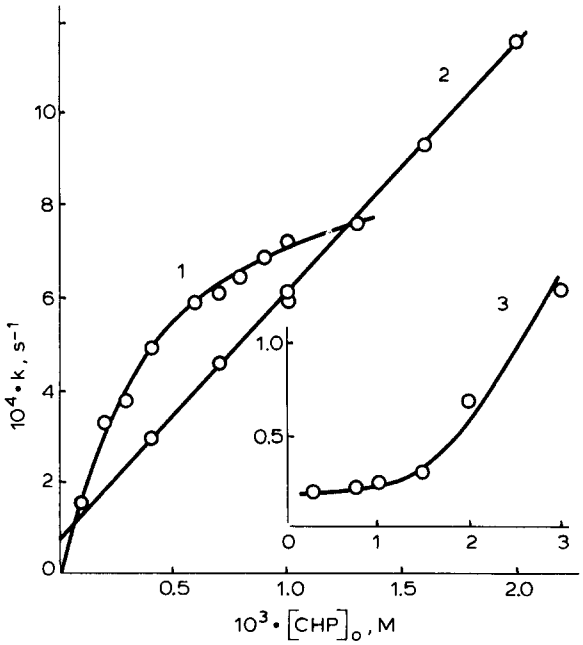

Fig. 7. The dependence of the effective rate constants of hemoprotein destruction in the buffer solution (pH 7.5, 15% glycerol) on $[CHP]_0$ at 31°C: 1, cytochrome *P*-450 LM-2 (2.5 μM); 2, myoglobin (6 μM), and 3, cytochrome b_5 (6.75 μM).

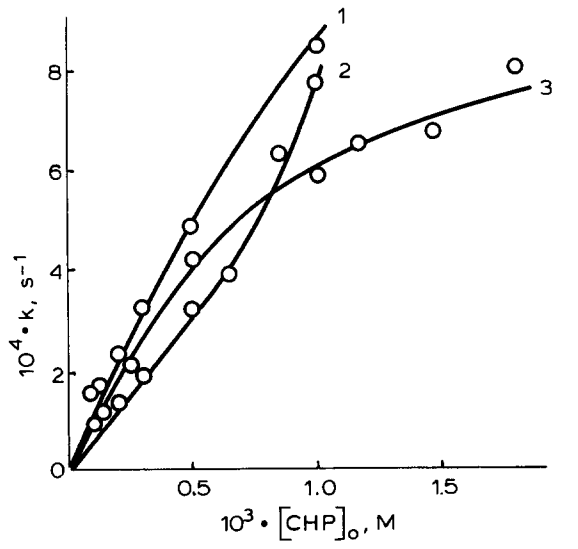

Fig. 8. The dependence of the effective rate constants of hemoprotein destruction in aerosol OT reversed micelles in octane on $[CHP]_0$ (0.25 M aerosol OT, 4.2% polar phase): 1, cytochrome *P*-450 LM-2 (1.86 μM); 2, myoglobin (5.97 μM), and 3, cytochrome b_5 (5.98 μM).

for cytochromes *P*-450 and b_5 have a hyperbolic character, whereas the effect of $[CHP]_0$ on k values for myoglobin in the mixed micelles is more complicated: a sharp increase in the rate constant is observed at $[CHP]_0 > 2$ mM.

Based upon our data and the findings of other authors [21–24], the transformation of the hemoprotein complexes with cumene hydroperoxide in aqueous solutions and in the reversed micelles of surfactants may be described by the following general scheme:

$$ROOH + \bar{L}Fe^{3+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \bar{L}Fe^{3+}{:}{:}\underset{\underset{H}{|}}{OOR} \quad (X) \qquad (1)$$

$$X \xrightarrow{k_{2'}} \bar{L}Fe^{2+} + RO_2^{\cdot} + H^+ \qquad (2')$$

$$X \xrightarrow{k_{2''}} L{\cdot}Fe^{2+}OH + RO^{\cdot} \qquad (2'')$$

$$X \xrightarrow{k_{2'''}} L{\cdot}Fe^{3+}OR + HO^{\cdot} \qquad (2''')$$

$$k_2 = k_{2'} + k_{2''} + k_{2'''};$$

$$r^{\cdot} = RO_2^{\cdot}, RO^{\cdot}, HO^{\cdot}$$

$$\bar{L}Fe^{3+} + r^{\cdot} \rightarrow \text{products of protein destruction} \qquad (3)$$

The complexes X in the absence of redox-active additivities are decomposed to form radicals via reactions 2′, 2″ and 2‴. The radicals $RO_2^{\cdot}$, $RO^{\cdot}$ and $HO^{\cdot}$ attack protoporphyrin IX and destroy it, which leads to a decrease in the Soret band intensity of hemoproteins. Reactions 2′, 2″ and 2‴ may be rate-limiting stages for the whole process, since the rate of reaction 3 is very high and the formation of complexes X also proceeds with a high rate [23,24].

In Table II, the effective constants of destruction rates for the three hemoproteins in aqueous solutions and in the reversed micelles of surfactants are compared. The use of mixed reversed micelles increases protein stability with regard to cumene hydroperoxide 4-fold in the case of cytochrome *P*-450 and 6-fold in the case of myoglobin. The stability of cytochrome b_5 towards cumene hydroperoxide in the mixed micelles approaches that in aqueous solution. The use of single reversed micelles of aerosol OT does not offer any advantages for hemoprotein protection from the

TABLE II

THE EFFECTIVE RATE CONSTANTS OF HEMOPROTEIN DESTRUCTION AT AN INITIAL CUMENE HYDROPEROXIDE CONCENTRATION OF 1.0 mM AT 31°C

Hemoproteins	$k \times 10^4$ (s^{-1})		
	Tris/H_3PO_4 buffer, pH 7.5, +15% glycerol	Reversed aerosol OT micelles	Reversed micelles of aerosol OT-Triton X-45 (1 : 1)
Cytochrome P-450 LM-2	7.3	8.5	1.6 *
Myoglobin	6.0	7.8	1.1
Cytochrome b_5	0.2	6.3	0.6

* The surfactant ratio was 1 : 2.

destructive action of cumene hydroperoxide. Moreover, in the reversed aerosol OT micelles cytochrome b_5 is decomposed much faster than in an aqueous medium. The mixed reversed micelles of an anionic and a neutral surfactant with predominant content of neutral detergent strongly protect the hemoproteins from destruction in reactions with cumene hydroperoxide. The observed effect may be assigned to either the decrease in the rate of cumene hydroperoxide conversion in the reversed micelles or to the inhibiting action of Triton X-45, a potential trapping agent for the active radicals $RO_2^{\cdot}$, $RO^{\cdot}$ and $HO^{\cdot}$.

Conclusion

Differing in their properties and functions, the hemoproteins, cytochrome *P*-450 and b_5, as well as myoglobin are easily entrapped into the reversed micelles of surfactants in octane. The process is accompanied by essential changes in protein structure as manifested in the blue Soret band shift and the decrease of its intensity. The protein transformation in the reversed micelles depends on factors characteristic of aqueous solutions of these proteins, i.e., buffer concentration, pH, temperature and the presence of stabilizing agents (e.g., glycerol), as well as on the factors characteristic of the micellar systems only: nature of surfactants, the $[H_2O]$/[surfactant] ratio and, finally, on the composition of the micelle nuclei. The protein nature is revealed by its stability in reversed micelles: cytochrome b_5 is more stable in single micelles, whereas the hydrophobic cytochrome *P*-450 is stable in mixed micelles (see Table I). This fact gives evidence of a strong effect of the lumenal surface on the solubilized proteins. This is supported by the alterations in the fluorescence spectra of cytochrome b_5 and in the CD spectra of myoglobin in reversed micelles as compared to the spectra of these hemoproteins in buffer solutions. A blue shift of the fluorescence maximum for cytochrome b_5 and quenching of fluorescence by water and Triton X-45 suggest that tryptophan residues of this protein are located near the lumenal surface of micelles.

The transformation of CD spectra of myoglobin in the ultraviolet region testifies to a considerable change in the secondary structure of this protein in micelles (see Fig. 6).

All three proteins retain their ability to react with cumene hydroperoxide in micelles. Cumene hydroperoxide is poorly soluble in water and it is unlikely that it penetrates to the micelles lumen. The high reaction rates of cumene hydroperoxide with hemoproteins in the micelles testify that the iron protoporphyrins of all three proteins are accessible to cumene hydroperoxide molecules. This suggests that the hydrophobic 'pocket' of the heme either faces the inner surface of micelles or even that it is located in the hydrophobic layer formed by the aliphatic fragments of surfactants.

The activation energy values for the cytochrome b_5 transformation in the reversed micelles indicate that, during transformation of this protein immediately after its incorporation into the reversed micelles, the hydrogen bonds are destroyed and hydrophobic interactions are weakened, since the activation characteristics of the process are comparable with the energy values for hydrogen bonds and hydrophobic interactions.

In the process of protein solubilization not only does protein affect the micelles, but these in turn affect the protein structure, altering its organization as compared to aqueous solutions. Based upon our data, a model of the system 'reversed micelle–solubilized protein' can be advanced. The main role is assigned to the interaction of the lumenal surface of a micelle with a protein-globule surface. The hydrophobic amino acid residues of

the protein surface are dissolved in a nonpolar layer of the reversed micelle, whereas the electrostatic interaction of polar 'heads' of surfactants and water molecules, structured by micelle, with polar sites of the protein surface allows the protein to be hold rigidly on the micellar surface. As a result, we observe a change in the secondary structure of the protein. The active sites of the protein (heme and its environment) either face the lumenal surface of micelles or are located in hydrophobic regions formed by nonpolar fragments of surfactants in the organic solvent.

We assume that the model considered closely imitates some features of natural membranes [13,25]. It should be noted that simple preparation of the reversed micelles, their stability in time and optical transparence make these the most simple and convenient model of biological membranes, allowing one to elucidate the fine structure of the native membranes and particularly their behaviour in the interaction with proteins of different natures, hydrophobic (cytochrome *P*-450) or water-soluble (myoglobin), since the protein nature determines the extent of its 'embedding' into the natural and model membranes.

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