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MAMMALIAN 3-OXOSTEROID Δ4-Δ5-ISOMERASE: A MEMBRANE-BOUND ENZYME

I. FLUORESCENCE STUDY OF THE RELATIONSHIP BETWEEN THE ENZYMATIC BINDING SITE, PHOSPHOLIPIDS, WATER AND IONS

JACQUES GALLAY, MICHEL VINCENT and ANNETTE ALFSEN

Laboratoire des Etats Lies Moleculaires, Equipe de Recherche du Centre, National de la Recherche Scientifique, U.E.R. Biomédicale des Saints Pères, Université René Descartes, 45 rue des Saints Pères, 75006 Paris (France)

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Summary

The microsomal membranes and the proteolipidic particles obtained by disruption of the microsomes by alkaline-earth ions at molar concentration have been compared by measuring the fluorescence properties of 1-anilinonaphthalene-3-sulfonate and naphthyl-1-phenylamine. The protein lipid arrangement of these two systems appears to be not essentially different. The study of fluorescence polarization of an hydrophobic probe (perylene) in function of Mg^{2+} concentration suggests a possible mechanism of disruption of the membrane by Mg^{2+} involving the strong structure-making effect of the ion.

The comparison of the fluorescence polarization changes of perylene and equilenine (a competitive inhibitor of the isomerase) with the ionic concentration indicates that there is no direct relation between the bulk lipidic phase and the enzymatic binding site properties.

Moreover, the emission of equilenine is completely quenched by I^-, in contrast with the napththyl-1-phenylamine and perylene probes, which clearly demonstrates the accessibility of the catalytic site to water molecules and ions.

Introduction

The enzymatic proton transfer from C_6 to C_4 on 3-oxosteroids is catalysed by isomerases (EC 5.3.3.1) which have been studied both in bacteria [1—5] and in mammalian organisms [6,7]. The catalysed reaction is the last step of the biosynthesis of biologically active hormones such as androstenedione and

Abbreviation: ANS, 1-anilinonaphthalene-8-sulfonate.

progesterone. Although a soluble enzyme has been found in the cytosolic fraction of rat liver [8], the mammalian isomerase of other tissues, especially in adrenal cortex, has been mainly located in the microsomal membranes [9,10]. It has been reported that the enzyme could be released from microsomal particles by addition of detergents such as deoxycholate [9] and Triton X-100 [11], however, no really satisfying solubilization procedure has been described.

We have shown that the microsomal membranes of beef adrenal cortex could be disrupted by the action of 1 M $CaCl_2$ [12,13]. The proteolipidic particles are enzymatically active and the specific isomerase activity is about the same or a little higher than in the microsomes. This fact permits to suppose a similar microenvironment for the enzyme in both systems. In order to describe this environment, a comparative study of microsomes and proteolipidic particles has been undertaken.

Fluorescence technique has been shown to be a powerful tool in the study of simple membrane model like lipid micelles and liposomes [14,15] and of more complex structures like biological membranes [16—19]. By combining quantum yield, maximum emission wavelength, quenching efficiency of protein tryptophans, fluorescence polarization measurements, it is possible to localize the probes either at nonpolar-polar interface (lipid-water) or in hydrophobic cores (likely the hydrocarbon regions of lipids) [20—22]. Thus, local informations on the microenvironment of the probes can be obtained during processes of conformational changes induced by perturbations such as temperature changes and ionic concentration [14—23].

Four fluorescent probes have been used in this work: amphiphilic (1-anilinonaphthalene-8-sulfonate (ANS)-), hydrophobic (naphthyl-1-phenylamine and perylene), and a steroid molecule (oestr 1,3,5, (10) 6,8-pentaene-3-ol,17-one: equilenine) which is a competitive inhibitor of the isomerase (Gallay, J., Vincent, M. and Alfsen, A., unpublished).

The present study is an attempt to describe the relation between the lipids, the isomerase and the aqueous solvent in the solubilized proteolipidic structures.

Materials and Methods

Enzyme preparation. Microsomes preparation has been previously described [13]. The proteolipidic particles are obtained after disruption of the microsomal membranes by 1 M $MgCl_2$. After centrifugation at 105 000 × *g*, 70—80% of the absorbance representing the non-sedimenting particles are eluted in a $E_v/E_{v\,o}$ = 3 on a Sepharose 2B column equilibrated with 0.2 M Tris · HCl (pH 7.2), 1 M $MgCl_2$. The lipid content of this material is a little lower than in the microsomes (lipid/protein = 43 and 53% (w/w), respectively) but the lipid composition is very similar [12]. The stability of the particles is very dependent of the ionic composition of the buffer and below 1 M $CaCl_2$ a precipitation occurs in function of time [13]. However, no precipitation can be observed during the few minutes needed for the fluorescence measurements.

Chemical reagents. 1-Anilinonaphthalene-8-sulfonate (ammonium salt) from Pierce, perylene (Merck 98% pure), naphthyl-1-phenylamine (Hopkins and Williams) and equilenine (Roussel UCLAF) where used without further

purification. Organic solvents were fluorimetric grade reagents. Ethylene diamine tetraacetic acid (EDTA) was Baker Chemicals reagent, analytical grade. All other chemicals were purchased from commercial sources.

Fluorescence measurements. Fluorescence spectra and intensities were determined with an absolute differential spectrofluorimeter Fica 55. Polarization of fluorescence measurements were carried out with the same instrument, fitted with polarizing filters in the emission and the excitation light path.

Quantum yield (ϕ) of bound dyes was calculated from the fluorescence intensities extrapolated at infinite protein concentration [25]. The quantum yield of ANS in butanol ($\phi = 0.56$) was taken as reference.

The number of dye sites and dissociation constants have been determined according to Klotz [26] and using the following equation:

$$\frac{P_0}{xD_0} = \frac{1}{n}\left(1 + \frac{\bar{K}_{D\ app}}{(1-x)D_0}\right)$$

where P_0 is the total protein concentration, D_0 is the total dye concentration, x is the fraction of bound dye, $\bar{K}_{D\ app}$ is the average dissociation constant of the dye, n is the number of dye sites.

By plotting P_0/xD_0 versus $1/(1-x)D_0$, n can be determined by the intersection with the ordinate. The intersection with the x axis gives $\bar{K}_{D\ app}$. The protein concentration was measured according to Lowry et al. [28]. The fraction of bound dye was obtained from the ratio of the observed fluorescence to that obtained when all this dye is bound at infinite protein concentration. A fraction of 3.3% (v/v) dioxane was used in experiments with naphthyl-1-phenylamine as probe. All the measurements have been obtained from difference spectra, the reference cell containing the dye in the same conditions. Fluorescence intensities were corrected for self-absorption of the incident light.

Fluorescence polarization degree (p) was calculated from the expression:

$$p = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the filters vertically and horizontally oriented, respectively.

The labelling of the proteolipid particles with perylene or equilenine was carried out according to Shinitzky et al. [23]. Very fine glass beads (Applied Science, Lab. Inc. 60/80 mesh), successively washed with sulfochromic acid, bidistilled water and methanol, were mixed with a methanol solution of each probe and dried up with mixing under N_2. The coated glass beads were equilibrated with the particles in 1 M $MgCl_2$ solution by stirring during 2 h at room temperature. The amount of bound perylene was calculated to be approx. 15 nmol/mg protein, for equilenine it was 180 nmol/mg protein. Corrections for the contribution of the scattered light to the polarization were done according to Shinitzky et al. [23], with an unlabelled reference solution of the same composition. The same method was used to determine the quantum yield of naphthyl-1-phenylamine bound to the proteolipidic particles in presence of 1 M Mg^{2+}.

Absorbance measurements. Absorbance was measured with a Cary 118 C spectrophotometer.

Results

(1) Binding of ANS and naphthyl-1-phenylamine to the microsomal membranes and to the proteolipidic particles. Influence of Mg^{2+} concentration on the binding parameters of the probes

The binding of ANS to both microsomes and to the proteolipidic particles produces a large enhancement of the dye fluorescence accompanied by a blue shift of the emission maximum. The data are summarized in Table I.

The effect of $MgCl_2$ on ANS fluorescence in microsomes has been studied (Fig. 1). It is apparent that the increase of the fluorescence intensity of the probe is associated with a 3-fold increase in the number of ANS molecules bound to the membranes and with a decrease of the apparent dissociation constant. No change in quantum yield ($\phi = 0.11$) can be detected.

Likewise, the number of ANS molecules bound to the proteolipidic particles is increased by Mg^{2+} concentration and $K_{D\ app}$ is decreased. However, at the highest concentration of Mg^{2+} used (0.1 M), the quantum yield of the bound dye increases from 0.12 to 0.22 and the maximum emission wavelength is further shifted from 482 to 477 nm. (Table I).

In the same conditions, the binding of naphthyl-1-phenylamine to both systems leads to an increase of the fluorescence intensity of the dye. The maximum emission wavelength is shifted to the blue. The data are presented in Table II. In both systems, the number of binding sites (n) and the dissociation constant ($\bar{K}_{D\ app}$), remain unchanged upon addition of Mg^{2+}. However, an increase of the quantum yield of the dye is observed in the proteolipidic particles at the highest Mg^{2+} concentration of 1 M. The maximum emission wavelength is simultaneously shifted from 430 to 412 nm. (Table II).

TABLE I

THE VARIATION OF THE BINDING PARAMETERS OF ANS IN THE MICROSOMES AND IN THE PROTEOLIPIDIC PARTICLES IN FUNCTION OF $MgCl_2$ CONCENTRATION

The protein concentration was 4.3 mg · l^{-1} in 20 mM Tris · HCl (pH 8.5). All experimental procedures are described in Materials and Methods. Excitation wavelength: 360 nm.

	$MgCl_2$ concentration (M)	Maximum wavelength	Quantum yield	Number of dye sites (nmol · mg^{-1})	Apparent dissociation constant $K_{D\ app}$ (μM)
Microsomes	0	483	0.11	45	125
	10^{-2}	483	0.11	125	60
Proteolipidic particles	0*	482	0.12	50	120
	$3.3 \cdot 10^{-3}$	482	—	—	—
	10^{-1}	477	0.22	200	83

* The zero point of Mg^{2+} concentration was obtained by adding 10^{-2} M EDTA in the cells.

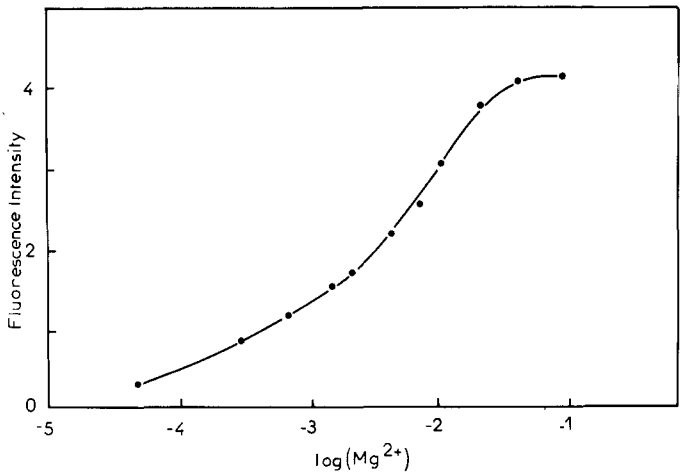

Fig. 1. The variation of the fluorescence intensity at 482 nm of ANS bound to microsomes, with the ionic concentration. Buffer: 20 mM Tris · HCl (pH 8.5). Excitation wavelength: 360 nm. Protein concentration: 0.05 mg · ml^{-1}. ANS concentration: 6.6 μM.

(2) Tryptophan quenching efficiency by ANS and naphthyl-1-phenylamine

Informations about the localization of the fluorophores in the membranes can be derived from observation on energy transfer between tryptophans in the membrane proteins and the bound probes.

Microsomes and particles show an intrinsic excitation maximum at 280 nm and an emission maximum at 335 nm, which are characteristic of tryptophans. When ANS is added to the microsomes or to the particles, the fluorescence at 335 nm is quenched and the emission at 482 nm increases, including an energy transfer from excited tryptophans to bound ANS. This interpretation is further supported by the existence of an isoemissive point at 425 nm [29] (Fig. 2a). The maximum quenching efficiency (Q) of ANS is strongly dependent of the

TABLE II

THE VARIATION OF THE BINDING PARAMETERS OF NAPHTHYL-1-PHENYLAMINE IN THE MICROSOMES AND IN THE PROTEOLIPIDIC PARTICLES IN FUNCTION OF $MgCl_2$ CONCENTRATION

Experimental conditions as in Table I except that 3.3% (v/v) dioxane was added. Excitation wavelength: 340 nm.

	$MgCl_2$ concentration (M)	Maximum wavelength	Quantum yield	Number of dye sites (nmol · mg^{-1})	Apparent dissociation constant $K_{D\ app}$ (μM)
Microsomes	0	430	0.34	500	25
	10^{-2}	430	0.34	500	20
Proteolipidic particles	0*	430	0.36	520	30
	$3.3 \cdot 10^{-3}$	430	—	—	—
	10^{-1}	430	0.36	490	35
	1	412	0.43	—	—

* The zero point of Mg^{2+} concentration was obtained by adding 10^{-2} M EDTA in the cells.

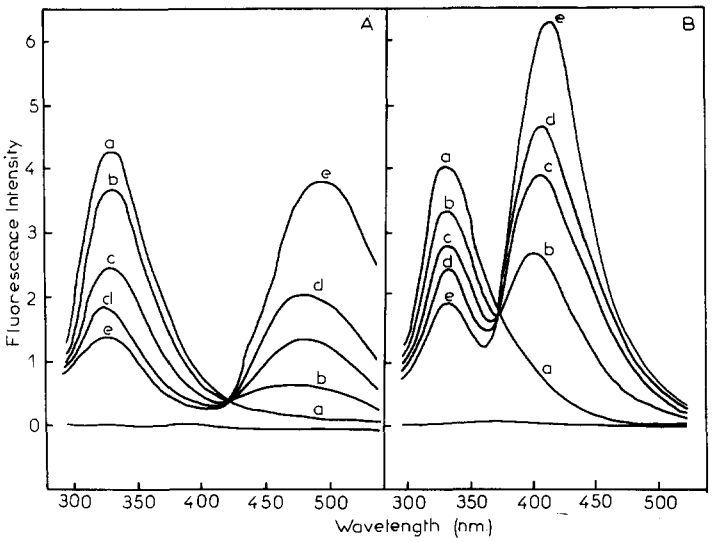

Fig. 2. (a) The energy transfer from the excited protein tryptophans to ANS bound to the proteolipidic particles. Buffer: 20 mM Tris · HCl (pH 8.5), 0.1 M $MgCl_2$. Protein concentration: 4.3 mg · l^{-1}. ANS concentration: a = 0, b = 2, c = 5, d = 15, e = 30 μM. Excitation wavelength: 280 nm. (b) The energy transfer from the excited protein tryptophans to naphthyl-1-phenylamine bound to the proteolipidic particles. Experimental conditions as in Fig. 2a, except 3.3% (v/v) dioxane was added in the cells. Naphthyl-1-phenylamine concentrations: a = 0, b = 1.4, c = 2.8, d = 5.2, e = 5.6 μM. Excitation wavelength: 280 nm.

Mg^{2+} concentration. An increase is observed which is parallel to the variation of the number of binding sites and the association constant of the probe (Table III).

In the case of naphthyl-1-phenylamine, a decrease of the tryptophan emission as well as an increase of the emission at 430 nm is observed. An isoemissive point is also observed at 372 nm (Fig. 2b). However, the maximum quenching efficiency is smaller than for ANS and independent of the ionic concentration (Table III).

TABLE III

THE VARIATION OF THE MAXIMUM QUENCHING EFFICIENCY OF PROTEIN TRYPTOPHANS BY ANS AND NAPHTHYL-1-PHENYLAMINE IN FUNCTION OF $MgCl_2$ CONCENTRATION

Comparison between microsomes and proteolipidic particles. Excitation wavelength: 280 nm. Protein concentration: 4.3 mg · l^{-1} in 20 mM Tris · HCl (pH 8.5).

	$MgCl_2$ concentration (M)	Maximum quenching efficiency of ANS	Maximum quenching efficiency of naphthyl-1-phenylamine
Microsomes	0	0.47	0.61
	10^{-2}	0.83	0.64
Proteolipidic particles	0*	0.46	0.62
	$3.3 \cdot 10^{-3}$	0.65	0.60
	10^{-1}	0.80	0.60

* The zero point of Mg^{2+} concentration was obtained by adding 10^{-2} M EDTA in the cells.

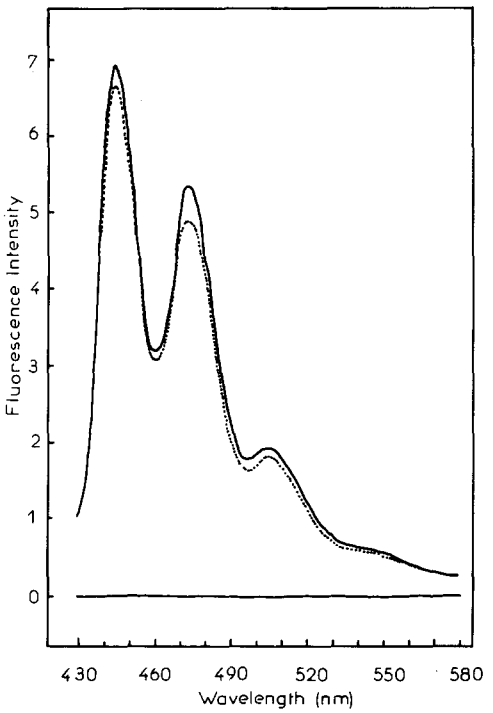

Fig. 3. Fluorescence spectrum of perylene bound to the proteolipidic particles. Buffer: 20 mM Tris · HCl (pH 8.5), 0.1 M $MgCl_2$. Protein concentration: 4.3 mg · l^{-1}. Excitation wavelength: 417 nm. ———, no I^- was added; ....., $[I^-]$ = 0.1 M. All others experimental conditions as in Materials and Methods.

(3) Fluorescence polarization of perylene

The spectrum of perylene bound to the proteolipidic particles is shown in Fig. 3. The fluorescence polarization of this dye has been measured in function

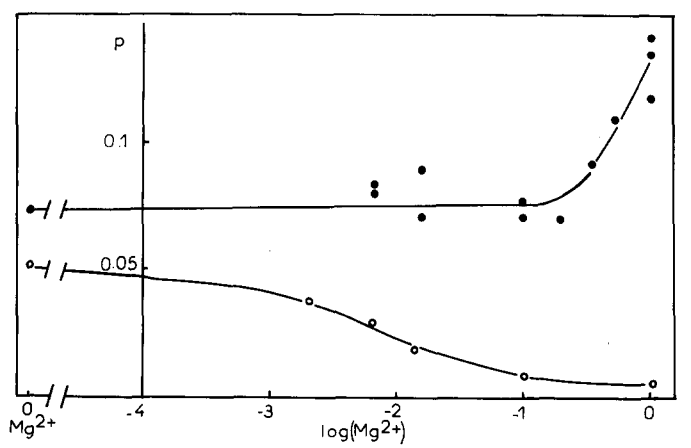

Fig. 4. The variation of the fluorescence polarization degree (*p*) of perylene (•) (excitation wavelength: 417 nm, emission: 445 nm) and equilenine (○) (excitation wavelength: 325 nm, emission: 365 nm) bound to the proteolipidic particles. Experimental conditions as in Materials and Methods. The zero point of Mg^{2+} concentration was obtained by adding 10^{-2} M EDTA in the cells. Protein concentration: 8.4 mg · l^{-1} (•) and 21 mg · l^{-1} (○).

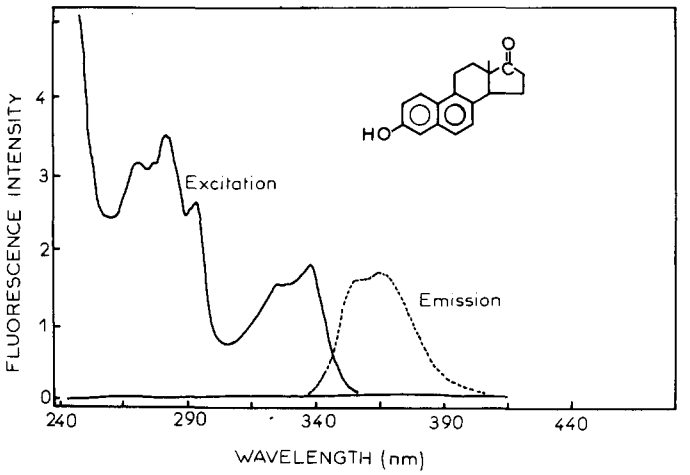

Fig. 5. Excitation (emission wavelength: 365 nm) and emission spectra (excitation wavelength: 335 nm) of equilenine bound to the proteolipidic particles. Buffer: 20 mM Tris · HCl (pH 8.5) 0.1 M $MgCl_2$. All others experimental conditions as in Materials and Methods. Protein concentration: 21 mg · l^{-1}

of Mg^{2+} concentration. It can be seen that at low Mg^{2+} concentration, p is equal to 0.075 and does not change up to 0.2 M Mg^{2+}. It increases beyond this concentration to reach a value of 0.14 at 1 M Mg^{2+} (Fig. 4).

(4) Fluorescence study of equilenine

Equilenine has been shown to be a competitive inhibitor of the mammalian steroid isomerase activity. The inhibition constant has a value of 38 μM when 5-androstene-3,17-dione is used as substrate [24]. The spectral properties of equilenine bound to the proteolipidic particle are reported in Fig. 5. When excited at 325 nm, a characteristic emission is observed at 365 nm. Moreover, a

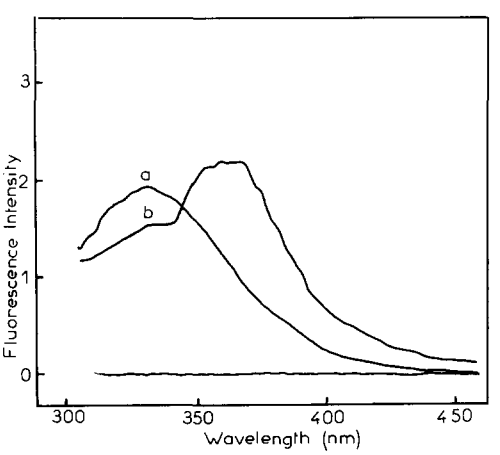

Fig. 6. The energy transfer from the excited protein tryptophans to equilenine bound to the proteolipidic particles. Protein concentration: 21 mg · l^{-1}. $MgCl_2$ concentration: 16.6 mM. Excitation wavelength: 280 nm. All others experimental conditions as in Materials and Methods. a, no equilenine was added; b, equilenine concentration: 3.6 μM.

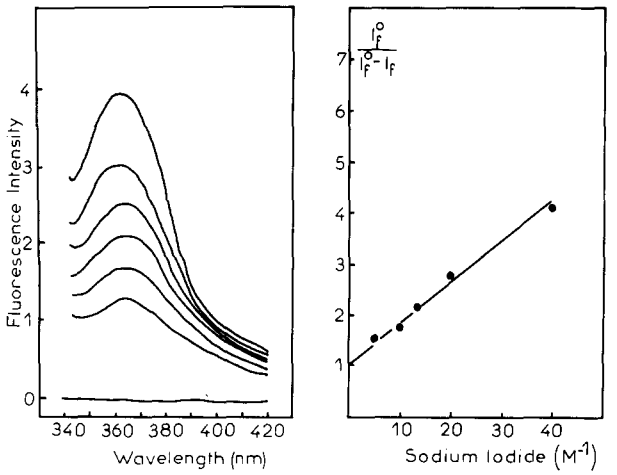

Fig. 7. (a) The fluorescence quenching by I^- of equilenine bound to the proteolipidic particles. Excitation wavelength: 325 nm. Experimental conditions as in Fig. 8. (b) Reciprocal plot of the fluorescence quenching of bound equilenine versus iodide concentration.

decrease of the fluorescence of the protein tryptophans of about 18% appears upon binding of equilenine to the proteolipidic particles. From the increase of the emission at 365 nm, it is apparent that an energy transfer occurs from excited tryptophans to the bound dye (Fig. 6). The fluorescence polarization degree (p) of this probe decreases from 0.051 to 0 in function of Mg^{2+} concentration. The midpoint effect is about 9 mM (Fig. 4).

(5) Naphthyl-1-phenylamine, perylene and equilenine fluorescence quenching by I^-

The quenching efficiency of I^- on equilenine fluorescence has been measured in order to obtain some indication about the accessibility of the isomerase binding site to water solvent and ions. It can be seen in Fig. 7 that a 100% maximum quenching efficiency is obtained at infinite iodide concentration. At 0.1 M I^-, a quenching of 55% is observed. In contrast, at this iodide concentration, the decrease of the fluorescence of the perylene is less than 10% (Fig. 3). A similar feature has been observed in the case of naphthyl-1-phenylamine.

Discussion

A strong structural similarity between intact microsomes and the proteolipidic particles resulting from fractionation of the membranes by divalent cations, appears from the data brought about by ANS and naphthyl-1-phenylamine fluorescence measurements. The similarity concerns the phospholipid-water and the phospholipid-water-protein interfaces. This ressemblance allows the use of the much smaller particles as a model for the study of the membrane organization and of the interactions between proteins, lipids, water and ions in the membranes.

From the study of ANS fluorescence in function of Mg^{2+} concentration, it

appears that because of its negative charge, ANS should be repelled by anionic sites in the membranes at basic pH [33]. If microsomal groups are shielded by the addition of cations, this repulsion is reduced and the binding equilibrium shifted bringing more ANS into a hydrophobic environment [18].

It is significant that a similar effect is observed for the fluorescence intensity, n and $\bar{K}_{D\ app}$ of ANS in the proteolipidic particles, which indicates that both systems are surrounded by a negative potential barrier. The fact that the binding parameters of naphthyl-1-phenylamine, an uncharged probe, are independent of ionic concentration, is in agreement with this interpretation [33].

An other set of comparative data is given by the values of the quantum yield and the maximum emission wavelength for the two probes which indicate a statistical microenvironment more polar for ANS than for naphthyl-1-phenylamine. This is in agreement with a localization of the charged probe at the lipid-water interface. The value of the maximum quenching efficiency of the protein tryptophans fluorescence and its dependence from Mg^{2+} concentration, suggest that ANS is also bound at the lipid-protein-water interface [20,22]. On the other hand, naphthyl-1-phenylamine is probably buried in the hydrophobic parts of the lipid regions as evidenced by its quantum yield and its maximum emission wavelength when in a bound state [22]. The existence of a maximum quenching efficiency of about 60% for this probe, suggests that some proteins may be partly embedded in the hydrophobic tails of the phospholipids [22].

These former data support the idea that the proteolipidic particles are subfractions of the intact microsomes presenting a similar organization. However, the two systems can be distinguished by their characteristic stability toward solvent perturbations by divalent cations [13]. At 1 M Mg^{2+} the microsomes are disrupted into the proteolipidic particles. At this concentration, the fluorescence polarization of perylene and the variation of the quantum yield of bound ANS and naphthyl-1-phenylamine, indicate a higher constrained state of the polar part of the phospholipids and of their hydrophobic core than in absence of ions. It has been shown that in phospholipidic lamellar systems or in liposomes [34,35], the state of hydration of the polar heads of the phospholipids modifies the mobility of the fatty acid chains and of the polar heads themselves [36,37]. In the present system, a comparable situation is likely to occur and the high concentration of Mg^{2+} might induce some "dehydrated state" around the polar heads, leading to an increase of rigidity of the hydrophobic backbone of the phospholipids. This ordering effect would be increased by the possibility of Mg^{2+} to produce bridges between adjacent negative charges.

Since in aqueous solution, the particles are only stable in the presence of molar concentration of divalent cations [13], i.e. highly organized surrounding water, the same type of medium may be present along the area of disruption inside the microsomes. These area could be the channels of ionic transport across the membrane in which the water molecules solvating the charged groups at these surfaces are strongly oriented. The competition for these water molecules between the charged groups and the Mg^{2+}, should be the determinant factor for the disruption of the microsomes initiated along these channels.

The variation of the state of mobility of equilenine as a function of ions is very different from that of perylene: the higher the Mg^{2+} concentration, the

higher the mobility of the bound equilenine. Two main hypothesis can be proposed to explain the differences between the properties of the bulk phospholipids and the microenvironment of the isomerase binding site. According to the first one, the isomerase binding site is located at the protein-water interface accessible to ions, independent of phospholipids. In this case, the phospholipid dependence of the enzymatic activity in the membranes [38] would result from a stabilization by the surrounding phospholipidic structure, of the active conformation of the protein. Alternatively, the enzymatic site may be in close contact to some phospholipids but the protein-lipid interactions modify the behaviour of the lipid moiety which possibly becomes more sensitive to ionic concentration. Whatever the hypothesis, the 100% quenching efficiency of equilenine fluorescence by I^-, compared to the absence of effect on the fluorescence of perylene and naphthyl-1-phenylamine, strongly suggests that the enzymatic binding site is accessible to ions and water molecules [39].

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