Biochimica et Biophysica Acta, 664 (1981) 134–142
Elsevier/North-Holland Biomedical Press

BBA 79261

MEMBRANE LIPID DYNAMICS AND ENZYMIC ACTIVITY IN BOVINE ADRENAL CORTEX MICROSOMES *

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(Received January 20th, 1981)

Key words: Fluorescence anisotropy; Fatty acid probe; 3-Oxosteroid $\Delta^5-\Delta^4$-isomerase; Lipid dynamics; (Adrenal cortex microsome)

The lipid dynamics of the adrenocortical microsomal membranes was studied by monitoring the fluorescence anisotropy and excited state lifetime of a set of anthroyloxy fatty acid probes (2-, 7-, 9- and 12-(9-anthroyloxy)-stearic acid (AP)) and 16-(9-anthroyloxy)palmitic acid (AS). It was found that a decreasing polarity gradient from the aqueous membrane interface to the membrane interior, was present. This gradient was not modified by the proteins, as evidenced by comparison of complete membranes and derived liposomes, suggesting that the anthroyloxy probes were not in close contact with the proteins. An important change of the value of the mean rotational relaxation time as a function of the position of the anthroyl ring along the acyl chain was evidenced. In the complete membranes, a relatively more fluid medium was evidenced in the C_{16} as compared to the C_2 region, while the rotational motion appeared to be the most hindered at the C_7-C_9 level. In the derived liposomes, a similar trend was observed but the mobility was higher at all levels. The decrease of the mean rotational relaxation time was more important for 12-AS and 16-AP. Temperature dependence of the mean rotational relaxation time of 2-AS, 12-AS and 16-AP in the complete membranes revealed the existence of a lipid reorganization occurring around 27°C and concerning mainly the C_{16} region. The extent to which the acyl chain reacted to this perturbation at the C_{12} level depended on pH. The presence of proteins increased the apparent magnitude of this reorganization and also modified the critical temperature from approx. 23°C in the derived liposomes to approx. 27°C in the complete membranes. Thermal dependence of the maximum velocity of the 3-oxosteroid $\Delta^5-\Delta^4$-isomerase, the second enzyme in the enzymatic sequence, responsible for the biosynthesis of the 3-oxo-Δ^4-steroids in the adrenal cortex microsomes, was studied. The activation energy of the catalyzed reaction was found to be low and constant (2–5 kcal · mol^{-1}) in the temperature range 16–40°C at pH 7.5, 8.5 and 9, corresponding to the minimum, intermediate and maximum rate, respectively. A drastic increase of the activation energy (20 kcal · mol^{-1}) was observed at temperature below 16°C at pH 7.5. A correlated change of the pK_{ES}^{app} as a function of temperature was detected; at 36°C pK_{ES}^{app} = 8.3 while at 13°C the value shifted to 8.7. The pH range of the group ionization was narrower at 13°C. In contrast with the behaviour of the 3β-hydroxy-Δ^5-steroid dehydrogenase, the 3-oxosteroid $\Delta^5-\Delta^4$-isomerase was apparently unaffected by the lipid reorganization at 27°C. It is suggested that this enzyme possesses a different and more fluid lipid environment than the bulk lipids.

* This paper is dedicated to Professor A. Rossi-Fannelli on the occasion of his 75th birthday.

Abbreviations: 2-AS, 2-(9-anthroyloxy)stearic acid; 7-AS, 7-(9-anthroyloxy)stearic acid; 9-AS, 9-(9-anthroyloxy)stearic acid; 12-AS, 12-(9-anthroyloxy)stearic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid; isomerase; 3-oxosteroid $\Delta^5-\Delta^4$-isomerase.

Introduction

The mammalian 3β-hydroxy-Δ^5-steroid dehydrogenase/3-oxosteroid $\Delta^5-\Delta^4$-isomerase system is concerned with the biosynthesis of most steroid hormones. It converts the 3β-hydroxy-Δ^5-steroids into

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their corresponding 3-oxo-Δ^4-derivatives [1]. Available evidence suggests that this conversion is mediated by two separate enzymes [2–5]. The first and rate-limiting reaction is catalysed by the 3β-hydroxy-Δ^5-steroid dehydrogenase (EC 1.1.1.145) [6,7]. The second step is catalysed by the 3-oxosteroid Δ^5–Δ^4-isomerase (EC 5.3.3.1). In mammalian steroidogenic cells, these enzymes are associated with either the mitochondria inner membrane or the endoplasmic reticulum [8–13]. They are 'integral' membrane proteins according to the Singer's classification [14], since they can be solubilized only by detergents [5, 15–17] and are inactivated by phospholipases [18]. Therefore, it was interesting to study the influence of lipid organization on the regulation of these enzyme activities [19]. The spatial organization of these two proteins inside the membrane could determine their response to the lipid environment [20].

In a preceding paper [21], a description of the enzymatic function of the 3β-hydroxy-Δ^5-steroid dehydrogenase in the microsome membranes from bovine adrenal cells was given. A correlation between the lipid dynamics (estimated by fluorescence depolarization of diphenylhexatriene) and the enzyme activity was found. The change in the activation energy of the enzyme-catalysed reaction occurred in the same temperature range (25–30°C) as the change in the membrane-lipid mobility. The pH dependence of the enzyme maximum velocity exhibited a different pattern above and below the transition temperature. Moreover, the apparent coupling between membrane-lipid 'fluidity' and enzyme kinetics was pH dependent. Some of the 3β-hydroxy-Δ^5-steroid dehydrogenase steroid substrates (i.e. dehydroepiandrosterone) amplified the lipid reorganization around 30°C.

In the present work, thermal and pH dependence of the isomerase maximum velocity were studied. The membrane lipids were probed using a set of anthroyloxy fatty acid derivatives [22–30]. The transverse location of the anthroyl ring allowed the examination of polarity and rotational diffusion at different levels throughout the membrane by measurements of the excited-state lifetimes and fluorescence anisotropy. Influence of temperature on the rotational relaxation times was also studied. This set of data complements the previous study on 3β-hydroxy-Δ^5-steroid dehydrogenase.

Materials and Methods

Chemicals. Steroids were a kind gift from Roussel-Uclaf (Romainville, France). Organic solvents were purchased from Merck (Darmstadt, F.R.G.) or Aldrich, (Beerse, Belgium). Anthroyloxy fatty acid derivatives were from Molecular Probes (Pleno, U.S.A.). Their purity was controlled by thin-layer chromatography using ethanol/H_2O (95 : 5, v/v) as solvent. Only one spot appeared under ultraviolet irradiation [25]. All other reagents were of the highest grade commercially available.

Microsomes preparation. Microsomal membranes were prepared as described previously [5], but no $MgCl_2$ was used in the homogenizing buffer and an additional centrifugation at 15 000 $\times g$ was performed before spinning the microsomal membranes.

Enzyme assay. Isomerase activity was measured as reported previously with 5(6)-androstenedione and 5(6)-estrenedione [31]. For K_m and V determination of 5(10)-estrenedione, 10-cm light-path cuvettes were used in a Cary 118 C spectrophotometer. Only 5(6)-androstenedione was used as substrate for the determination of the effect of temperature on the enzymatic activity. Maximum velocity was estimated from extrapolation at infinite substrate concentration according to Lineweaver and Burk. Temperature was controlled inside the assay cuvette by a thermistor probe (YSI Instruments).

Liposomes preparation. Lipid extraction from microsomal membranes was carried out according to the method of Folch et al. [32] modified by Rouser and Fleischer [33]. Multilamellar vesicles were obtained after evaporation of the chloroform under nitrogen. The samples were suspended in 50 mM Tris-HCl, pH 8.5, 0.1 M NaCl, by vortexing.

Labelling of membranes with anthroyloxy fatty acid derivatives. Microsomal membranes were incubated 1 h at room temperature with 1 to 2 μl of a 1 mg/ml tetrahydrofuran solution of 2-, 7-, 9-, 12-(9-anthroyloxy)stearic acids and 16-(9-anthroyloxy)palmitic acid. Probe/phospholipid molar ratio was approx. 1/160. The measurement of the lipid content of the microsomal membranes has been previously described [18,34].

Fluorescence measurements. Excited state lifetime measurements were performed on a time-correlated single photon instrument as described previously

[21]. Deconvolution was carried out using the method of Valeur [35] followed by a non-linear least-square regression. Mean lifetimes were calculated using the expression:

$$\langle\tau\rangle = \int_0^\infty t \cdot i(t)\,dt \Big/ \int_0^\infty i(t)\,dt$$

$$= \frac{\sum_i a_i \tau_i^2}{\sum_i a_i \tau_i}$$

with $i(t)$ representing the decay of the total emission, τ_i the lifetime of the i-th component and a_i the corresponding amplitude. Mean lifetime values were used in the computation of the rotational relaxation times.

Fluorescence polarization measurements were performed with a T-format SLM 8000 apparatus equipped for photon-counting [36] and thermostatically controlled with a water-circulating device. The electronic device of this system allows background substraction simultaneously on each optical channel when needed. Temperature scans were performed by continuous heating and cooling of the sample at the approximate rate of 10–13 K/h. Temperature (±0.5°C) was measured directly in the sample cuvette with a YSI probe. Excitation wavelength was set at 365 nm with 2 or 4 nm bandpass. Schott filters KV 408 were used on each optical channel at the emission sides.

Fluorescence anisotropy (r) was obtained from intensity measurements using:

$$r = (I_{VV}G - I_{VH})/(I_{VV}G + 2I_{VH})$$

where I_{VV} is the intensity measured when polarizer and analyzer prisms are in the vertical position, and I_{VH} is the intensity when the analyzer prism is in the horizontal position. G is a correction factor given by the ratio of the vertical to the horizontal components when the excitation light is polarized in the horizontal direction. Suspensions having an absorbance of less than 0.13 at 450 nm were used.

Rotational relaxation times were calculated using the equation of Perrin [37]:

$$(r_0/r) - 1 = 3\tau/\rho$$

where r is the anisotropy, r_0 the intrinsic anisotropy, τ the excited state lifetime and ρ the rotational relaxation time. This parameter corresponds to the time it takes for molecules to rotate through an angle θ, such as $\cos\theta = e^{-1}$. This equation is fully applicable to non-spherical fluorophores only if their absorption and emission oscillators are parallel. Since this is not the case for the anthroyloxy derivatives, this parameter is a harmonic mean between in- and out-of-plane rotations [38–41].

To construct Arrhenius plots of $\log\langle\rho\rangle$ versus T^{-1}, excited state lifetimes of the probes were measured at five temperatures between 4 and 40°C. Linear thermal dependences of $\langle\tau\rangle$ were observed and extrapolated values were used.

Results

Determination of the kinetic parameters of isomerase for different substrates

K_m and V of three steroid substrates (5(6)-androstenedione, 5(6)-estrenedione and 5(10)-estrenedione) were determined (Table I). The best substrate 5(6)-androstenedione was used in the other measurements.

Effect of temperature and pH on the enzymatic activity

Arrhenius plots for isomerase activity with 5(6)-androstenedione were constructed at pH 7.5, 8.5 and 9 (Fig. 1). The apparent activation energy of the isomerization reaction is low (2–5 kcal · mol^{-1}) whatever the pH for temperatures above 16°C ± 1. At pH 8.5 the Arrhenius plot is a straight line in contrast with the observation for 3β-hydroxy-Δ^5-steroid dehydrogenase. At pH 7.5 a break is observed at 16°C ± 1 and below this temperature the apparent activation

TABLE I

KINETIC PARAMETERS OF THE 3-OXOSTEROID Δ^5–Δ^4-ISOMERASE FROM ADRENAL CORTEX MICROSOMES

Substrates	K_m (μM)	V (nmol · min^{-1} · mg^{-1})
5(10)-Estrenedione	280–350	35–41
5(6)-Estrenedione	87–111	300
5(6)-Androstenedione	103	445

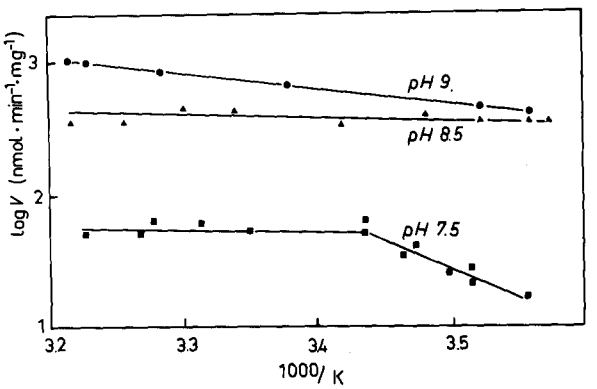

Fig. 1. Arrhenius plot of the isomerase maximum velocity at pH 7.5 (■), 8.5 (▲) and 9 (●). Each value corresponds to the extrapolation according to Lineweaver-Burk of seven initial velocity measurements performed in duplicate.

energy becomes 20 kcal · mol^{-1}. The Dixon plots below and above the break as a function of pH are different with a $pK_{ES}^{app} \simeq 8.3$ at $t = 35°C$ and $pK_{ES}^{app} \simeq 8.7$ at $t = 13°C$ (Fig. 2).

Fluorescence studies of the microsomal membranes and derived liposomes

Availability of anthroyloxy fatty acid derivatives gives the opportunity to study with a sensitive technique the possible existence of environmental and motional differences throughout the membrane lipid regions.

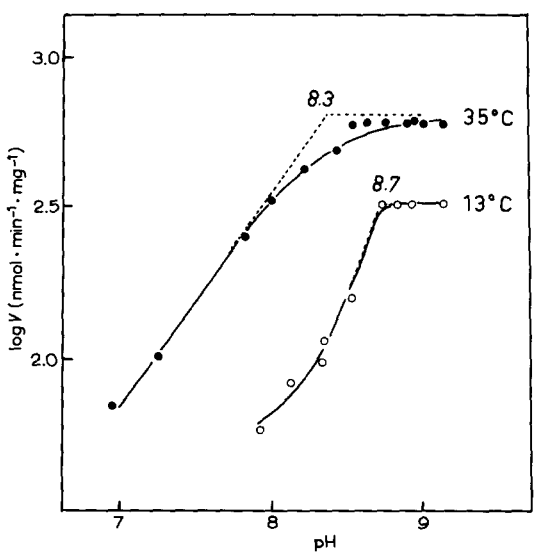

Fig. 2. Dixon logarithmic plot of the isomerase maximum velocity as a function of pH. Temperature dependence of pH was accounted for.

(a) Polarity gradient. The fluorescence characteristics, such as the lifetime of the excited state of the anthroyloxy fatty acid derivatives, are sensitive to the solvent relaxation or polarity. Thus a measure of the lifetimes of these probes permits an estimation of the relative polarity at the different membrane levels.

In the microsomal membranes, the experimental fluorescence decay curves of all probes are best fitted by a double exponential model (Table II). So are also the decay curves obtained for these probes in the liposomes prepared from microsomes (Table III).

Whatever the cause of such a behaviour in both types of membranes, the mean lifetime of the excited state (which reflects essentially the longest component of the decay) increases from the 2-AS to the 12-AS and then remains constant, indicating a polarity gradient from the 2 to the 12 position along the fatty acyl chain (Fig. 3). The proteins have no significant influence on the respective value of the fluorescence decay components (Table III). The variation in the values of the amplitudes of each component accounted for the small difference in the values of the mean lifetimes of 12-AS and 16-AP (Table III).

(b) Mobility gradient throughout the microsomal membranes and the derived liposomes. The calculated values of the mean rotational relaxation time of the five probes demonstrate that the anthroyl ring experiences changes in rotational rates when linked at different positions on the acyl chain of the fatty acid. As shown in Fig. 4, the rotational motion of the membrane lipids is hindered in the 7 and 9 region of the hydrocarbon chains. The mobility gradient pattern is preserved after removal of the proteins in the obtained liposomes. However the effect of the proteins is not the same at the different membrane levels: a greater perturbation is visible in the 12–16 region (Fig. 5). The variation of $\langle\rho\rangle$ is mainly due to a change in the anisotropy value (Table IV). 4-androstenedione was used in a concentration range of 6–70 μM (steroid/phospholipid $\simeq$ 0.08–0.84) in the same order of magnitude as in the enzymatic assays. This steroid has no effect on the values of $\langle\rho\rangle$, measured at the different levels of the membrane.

(c) Effect of the temperature on the lipid mobility at different levels of the hydrocarbon chain in the

TABLE II

FLUORESCENCE DECAY PARAMETERS AT 23–25°C OF THE ANTHROYLOXY FATTY ACID DERIVATIVES INCORPORATED IN ADRENOCORTICAL MICROSOMAL MEMBRANES

The best values of the mean weighted residues (MWR) are given.

Probe	Monoexponential model		Biexponential model					
	τ (ns)	MWR	τ_1 (ns) ± S.D.	τ_2 (ns) ± S.D.	A_1 ± S.D.	A_2 ± S.D.	MWR	$\langle\tau\rangle$ (ns) ± S.D.
2-AS	6.7	17.4	8.5 ± 0.3	3.7 ± 0.9	0.47 ± 0.05	0.53 ± 0.05	2.5	6.9 ± 0.1
7-AS	8.5	11.8	9.7 ± 0.1	4.2 ± 0.9	0.60 ± 0.04	0.40 ± 0.04	1.8	8.5 ± 0.2
9-AS	9.3	18.2	10.9 ± 0.3	3.9 ± 1.2	0.62 ± 0.03	0.38 ± 0.03	2.6	9.6 ± 0.1
12-AS	11.2	18.7	12.8 ± 0.5	4.4 ± 1.7	0.56 ± 0.06	0.44 ± 0.06	2.7	11.0 ± 0.4
16-AP	11.2	16.0	12.4 ± 0.5	3.7 ± 0.7	0.61 ± 0.03	0.39 ± 0.03	2.5	11.1 ± 0.5

TABLE III

FLUORESCENCE DECAY PARAMETERS AT 23°C OF THE ANTHROYLOXY FATTY ACID DERIVATIVES INCORPORATED IN LIPOSOMES PREPARED FROM ADRENAL CORTEX MICROSOMES

Probe	Monoexponential model		Biexponential model					
	τ (ns)	MWR	τ_1 (ns)	τ_2 (ns)	A_1	A_2	MWR	$\langle\tau\rangle$ (ns)
2-AS	7.1	7.9	8.0	3.6	0.61	0.39	2.8	7.0
7-AS	8.6	7.4	9.5	3.7	0.72	0.28	3.3	8.7
9-AS	10.2	8.1	11.1	3.6	0.74	0.26	3.4	10.3
12-AS	11.5	19.4	12.7	2.1	0.64	0.36	2.7	11.6
16-AP	11.6	9.5	12.7	3.6	0.72	0;28	2.7	11.8

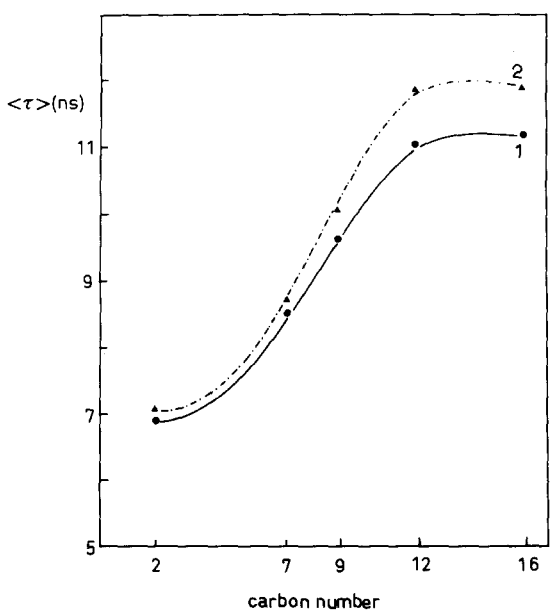

Fig. 3. Variation of the mean lifetime of the excited state ($\langle\tau\rangle$) of the anthroyloxy fatty acid probes as a function of the position of the anthroyl ring. 1 (•), complete membranes; 2 (▲), derived liposomes. Values are obtained after background and scattering substration. Temperature: 23–25°C. Standard deviations were in the range of 0.1–0.5 ns.

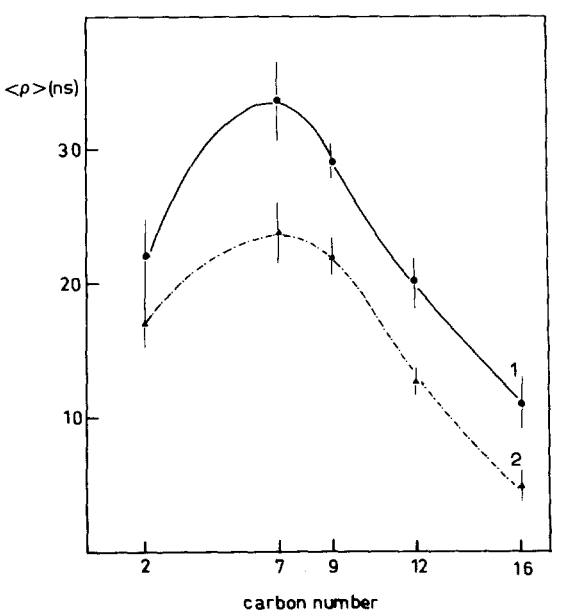

Fig. 4. Variation of the mean rotational relaxation time ($\langle\rho\rangle$) of the anthroyloxy fatty acid probes as a function of the anthroyl ring position. 1 (•), complete membranes; 2 (▲), derived liposomes. Values are obtained after background and scattering substraction. Temperature: 23–25°C.

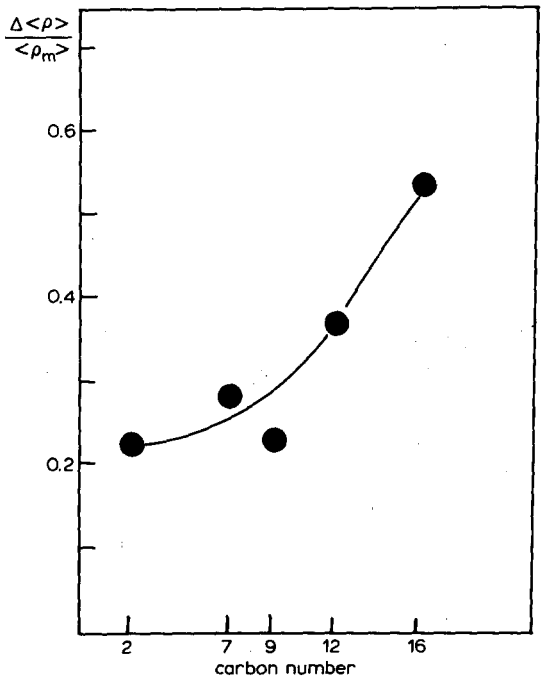

Fig. 5. Relative difference $\Delta\langle\rho\rangle/\langle\rho_m\rangle$ between the values of the rotational relaxation time obtained in complete membranes ($\langle\rho_m\rangle$) and derived liposomes ($\langle\rho_l\rangle$). $\Delta\langle\rho\rangle = \langle\rho_m\rangle - \langle\rho_l\rangle$.

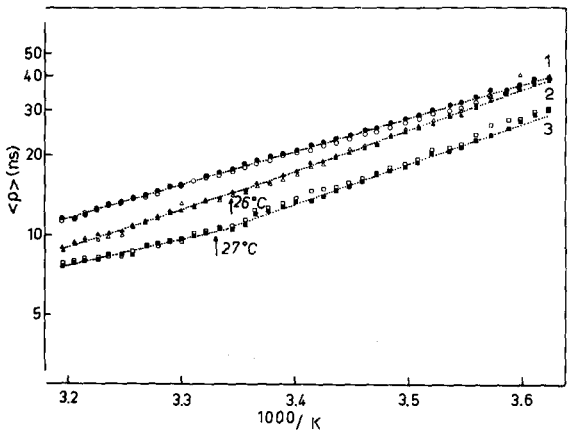

Fig. 6. Arrhenius plot of the mean rotational relaxation time ($\langle\rho\rangle$) as a function of $1/T$ in the complete membranes pH 7.5. Probes: 1 (●), 2-AS; 2 (▲), 12-AS and 3 (■), 16-AP. Open symbols refer to experiments in presence of 4-androstenedione (70 μM). Protein concentration approx. 0.13 mg · ml^{-1}.

membranes and in the liposomes. Rotational relaxation times of 2-AS, 12-AS and 16-AP were measured as a function of temperature at two pH (7.5 and 8.65) and are given in Figs. 6 and 7. Logarithmic representations of $\langle\rho\rangle$ vs. $1/T$ for 2-AS give straight lines. Linear regressions from 3 to 30°C and from 30 to 40°C do not indicate a difference higher than 7% between the slopes (correlation coefficient: 0.9995). In contrast, the curve for 16-AP can not be fitted by a single straight line. Linear regressions from 3 to 30°C and from 30 to 40°C indicate a difference in the slope values of about 40% (36 to 43%) (correlation coefficients: 0.9957 and 0.9954). The two straight lines intercept at 27°C. The results are the same at the two pH values studied.

With 12-AS, the difference between the slope values is only 9% at pH 7.5 (correlation coefficients: 0.9989 and 0.9939) but 20% at pH 8.65 (correlation

TABLE IV

COMPARISON OF THE ANISOTROPY VALUES (r) AT 23–25°C OF THE ANTHROYLOXY FATTY ACID PROBES IN ADRENOCORTICAL MICROSOMAL MEMBRANES AND IN MICROSOMAL EXTRACTED LIPID LIPOSOMES

Figures are presented as mean ± S.D. for at least 10 determinations. Rotational relaxation times are calculated using mean lifetime values of Table II and III. Intrinsic anisotropy values (r_0) were obtained at −50°C in propylene glycol at 365 nm.

Probe	r_0	Microsomes		Derived liposomes	
		r	$\langle\rho\rangle$ (ns)	r	$\langle\rho\rangle$ (ns)
2-AS	0.269	0.139 ± 0.009	22 ± 3	0.121 ± 0.009	17 ± 3
7-AS	0.265	0.151 ± 0.006	34 ± 3	0.127 ± 0.007	24 ± 2
9-AS	0.273	0.138 ± 0.003	29 ± 1	0.114 ± 0.005	22 ± 1
12-AS	0.271	0.103 ± 0.005	20 ± 2	0.071 ± 0.002	12 ± 1
16-AP	0.261	0.065 ± 0.006	11 ± 2	0.033 ± 0.001	5 ± 1

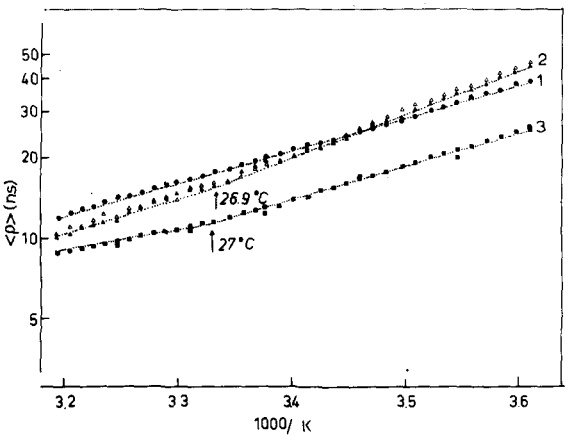

Fig. 7. Arrhenius plot of the mean rotational relaxation time ($\langle\rho\rangle$) as a function of $1/T$ in the complete membranes at pH 8.65. Probes: 1 (•), 2-AS; 2 (▲), 12-AS and 3 (■), 16-AP. Open symbols refer to experiments in the presence of 4-androstenedione (70 μM). Protein concentration approx. 0.13 mg · ml^{-1}.

coefficients: 0.9956 and 0.9857) with an intercept at about 26–27°C in both cases.

In the extracted lipid liposomes, the results for 2- and 12-AS can be described by a single straight line, the slope values being no more different than 6% (correlation coefficients: 0.9988 and 0.9996 for 2- and 12-AS, respectively). For 16-AP, the curve can be fitted by two straight lines with an intercept at approx. 23°C (correlation coefficients: 0.9977 and 0.9954). The difference in the slope values is 20% (Fig. 8).

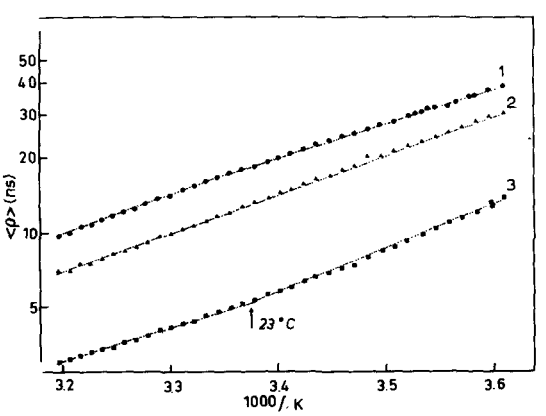

Fig. 8. Arrhenius plot of the mean rotational relaxation time ($\langle\rho\rangle$) as a function of $1/T$ in the derived liposomes at pH 8.5. Probes: 1 (•), 2-AS; 2 (▲), 12-AS and 3 (■), 16-AP. Lipid concentration: 0.07 mg · ml^{-1}.

Discussion

The present results have shown that in the microsomes obtained from endoplasmic reticulum of adrenal cortex, the fluorescent probes anthroyloxy fatty acid derivatives are exposed to a heterogeneous surrounding along the hydrocarbon acyl chain.

In contrast with anthracene, the excited-state lifetimes of 9-anthroic acid and its esters are sensitive to solvent polarity and relaxation [42,43]. Therefore, using the anthroyloxy probes, 'polarity' variations along the lipid acyl chains might be detected. Indeed, the variation of the mean lifetime values of the probes indicates a decreasing polarity gradient throughout the membrane, unaffected by the presence of the proteins. This gradient is not the result of an intrinsic property of the probes themselves, since in organic solvents the excited state lifetime values decreased from the 2-AS to the 12-AS and then increased for 16-AP (unpublished results). Thus, the variation of the mean lifetime values of the probes observed in the membranes reflects the influence of the surrounding medium on the excited-state properties of the probes.

However, a more detailed analysis of the data reveals that in the complete membranes, as well as in the derived liposomes, the decay curves of the total fluorescence intensity were best fitted by a double exponential model. The values of each lifetime component were identical in both systems within experimental precision, indicating that the double exponential curve was not the result of a protein-mediated heterogeneity of emitting sites. This result is different from the data previously obtained with diphenylhexatriene [21] and suggests that the anthroyloxy probes are actually located in the bulk lipids of the membrane, while diphenylhexatriene could be partitioned between the bulk and 'boundary' lipids.

Thus, if a polarity gradient along the acyl chain can be inferred from the variation of the mean lifetime, another kind of heterogeneity of the emitting sites might be inferred from the existence of two excited-state lifetimes for each probe, whatever the level in the membrane. This heterogeneity is not due

to the proteins and might represent a lipid/probe microdomain [44].

A mobility change along the acyl chain of the lipid, in the complete membranes and in the liposomes, was also demonstrated by monitoring the fluorescence polarization and lifetime of these probes.

A mobility hindrance appeared at the C_7–C_9 position. The C_{12}–C_{16} region is more fluid. However, in the derived liposomes, the mobility was higher at all levels tested, and the more important difference was observed in the C_{12}–C_{16} region. Two elements contributed to the existence of this mobility change in the complete membranes: the prominent presence of unsaturated acyl chains [45] and the presence of proteins, which reduced the lipid mobility in the deepest hydrophobic region of the membranes. In fact a mobility hindrance at the C_7–C_9 levels has also been evidenced in pure unsaturated phospholipids using similar probes [28]. However, these mobility characteristics must be interpreted with care, since recent observations have indicated that each of these probes behaves differently in an isotropic medium of liquid paraffin (unpublished results). These behaviours can be accounted for by the different hindrances exerted by the acyl chain on the in- and out-of-plane rotations of the anthroyl ring at the different positions along the acyl chain [38–41].

Temperature dependence of the rotational motion of these probes in the complete membranes differs with the position of the anthroyl ring along the chain. The data revealed the existence of a reorganization of the lipid region of the membranes occurring near 27°C and concerning mainly the deepest hydrophobic part of the lipid acyl chains, where the protein perturbation was also more pronounced. The pH had no effect on the fluorophore in the polar region and, surprisingly, affected the hydrophobic part of the membrane lipids: at pH 8.65, the effect of the thermal reorganization of the lipids at 27°C on the rotational motion of 12-AS was more pronounced than at pH 7.5.

The absence of proteins in the derived liposomes modified the temperature dependence of the probe motion; an increase of the slope values of the Arrhenius plot of $\log \langle \rho \rangle$ vs. T^{-1} (which reflects the activation energy of rotation) was observed in liposomes as compared to the complete membranes. This increase was greater for 16-AP than for the other probes. These observations confirm the effect of the proteins as slowing down the lipid motion in the nanosecond time scale, mainly in the deepest portion of the acyl chain.

The picture which emerges from the results presented here and from our previous data [21] is one of a membrane with a polar area relatively insensitive to pH and slightly perturbed by the proteins, in so far as motion in the nanosecond time domain is considered. In contrast, the hydrophobic deepest part of the fatty acyl chains exhibits a decreased mobility due to the proteins. This hydrophobic part experiences a change in organization as a function of temperature around 27–30°C. This change is sensitive to pH and, as has been reported previously [21], is amplified in the presence of some of the diffusible steroids.

The nature of the molecular reorganization leading to this dynamic change is unknown up to now, but because of the high unsaturation degree of the phospholipids, a melting of the acyl chain in this temperature range is precluded. On the other hand, for similar membranes with a high content in unsaturated phosphatidylethanolamine [46,47], a lipid molecular organization different from the bilayer phase has been suggested [48]. In fact, it has been recently reported that bilayer-nonbilayer reorganization of membrane lipids primarily affected the acyl chain motion and not the polar head group [49]. Other reports have also pointed out the effect of pH on this kind of reorganization [50].

What is then the influence of these membrane structural changes upon the membrane-associated enzymatic functions? In contrast to the 3β-hydroxy-Δ^5-steroid dehydrogenase, which was affected to some extent by these events, isomerase, the following enzyme in the metabolic pathway of the steroid hormone biosynthesis, does not seem to be under the influence of such lipid changes.

Thus in these membranes the enzyme responses to lipid perturbation appear diversified. This conclusion is strengthened by a recent report concerning the 21-hydroxylase multienzyme system [51], describing a sharp change in the activation energy of the catalytic reaction at about 25–26°C.

The reason for such a diversification could be the existence of different lipid microdomains [52,53] around the various proteins and the differences in the structure of the enzymatic proteins themselves.

Acknowledgements

We are indebted to L.L. Lewis for careful correction of the english. This work has been supported by C.N.R.S., E.R. 06 0064, INSERM U. 221 and D.G.R.S.T. A-C No. 77-7-1270. One of the authors (A.A.) wishes to thank Professor A. Rossi-Fannelli for his hospitality in the Istituto di Chimica Biologica.

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