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# Anticushing Drug Metyrapone Exhibits Specific Interactions with Serine Containing Systems. A Possible Molecular Target?

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## ABSTRACT

*Metyrapone* (2-*methyl*-1,2-*di*-3-*pyridyl*-1-*propanone*) *is a drug largely used as inhibitor of glucocorticoid synthesis. Although its binding to various proteins has been well indentified, its accurate molecular mechanism of action remains unknown. Therefore, the interactions of metyrapone* (*MET*) *with various membrane components such as phospholipids, cholesterol, their corresponding polar heads and a model serine containing peptide have been investigated by NMR and ESR methods. It was found that neither cholesterol nor most of the phospholipids tested, nor dimyristin exhibit any interaction with MET, except phosphatidylserine* (*DMPS*). *Furthermore, only serine bearing polar head* (*O-phosphoserine*) *showed an association with MET* (*stoechiometry* 1:1, *Kd* = 3200*M*-1). *As similar observations were also performed on serine alone and in the presence of the serine containing model peptide,* (*NASDSDGQDL*), *a possible implication of these interactions in the binding recognition of MET on serine-containing active site was finally tested and discussed.*

***Keywords*:** *Metyrapone, Membranes, Serine, NMR, ESR, Interactions*

## 1. Introduction

In the clinical field, metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone MET), an inhibitor of glucocorticoid synthesis [1], is widely used to treat the Cushing disease [2,3]. Since high blood corticosterone levels have been linked to a depressive mood [4], metyrapone is also used to treat Cushing related mental disturbances [5] and major depression [6-9]. However, metyrapone exhibits other properties highlighted by preclinical studies that can not be explained by its action on the corticotrope axis. At the physiological level, metyrapone injection is followed by a slight hypothermia [10] not reversed by glucocorticoid supplementation [11], a decreased locomotion [12], an enhanced arousal [13] together with biological signs of stress [14]. The latter does not mask the protective effect of metyrapone against psychological stressors [15,16] and physiological stressors such as brain ischemia [17,18] and excitotoxicity [19]. At the cell level, metyrapone modify the energy metabolism [10] with a decreased use of glucose [20-21] and alters the mitochondrial functioning [22]. Lastly, metyrapone also modify the detoxification as it modifies the expression of numerous cytochrome P450 such as CYP1A1 and CYP 3A [23].

Besides these integrated levels, the molecular support, *i.e.* the identification or the molecular target, still failed. Metyrapone profoundly modify the steroid metabolism through its inhibitory activity on the 11$\beta$ hydroxylase (CYP11B1), [1], 17$\beta$ hydroxysteroid deshydrogenase (17$\beta$-HSD) [24] and the 11$\beta$-HSD [25]. Metyrapone also inhibits P450 [26] in numerous species [27]. Its inhibitory effect is not specific as it targets the CYP11B1 [28] and the CYP3A [29], but also other enzymes such as lipooxygenase [30], guanylate cyclase [31] or nitric oxide synthetase as a member of cytochrome P450-like hemoprotein [32]. Some works have focused on the mechanisms by which metyrapone interacts with P450. Its fixation on P450 is independent from the redox state of the enzyme and the availability of oxygen [33,34] and does not modify the conformation of the protein P450A3 [35,36]. Another mechanisms suggested was the possible

 

implication of membrane components, phospholipids or cholesterol [37,38]. The investigation of this hypothesis was the starting point of the present study. NMR and ESR experiments were recorded on synthetic multilayers of phospholipids and sphingolipids, their building blocks (polar head groups and acyl chain backbone) and cholesterol. This selection led to identify a potential target and to finally observe the interactions of MET with a model peptide containing this molecular target, serine.

## 2. Materials and Methods

### 2.1. Chemicals

Dymiristoyl phosphatitdylcholine (DMPC), dymiristoyl phosphatidylethanolamine (DMPE), dymiristoyl phosphatidylserine (DMPS), dimyristin, bovine brain sphingomyelin, ceramids, galactocérébrosides, dipalmitoylphosphatidylglycerol DPPG, phosphorylcholin O-phosphoserine, serine, O-phosphocolamine, ethanolamine were purchased from Sigma-Aldrich (La Verpillère, France) and used without purification.

The model peptide N10L: Asn-Ala-Ser-Asp-Ser-Asp-Ser-Gly-Gln-Asp-Leu (NASDSDGQDL) was purchased from NeoMPS, Stasbourg, France. Proton attribution, and purity control were performed in D2O by using classical NMR technics (TOCSY, HMBC, HMQC, nOesy [39]) at 298 K. No stable conformation of the peptide could be identified at this step.

MET was from Sigma. Purity control and proton assignment were obtained by classical NMR methods (1H, COSY, HMBC, HMQC [39]), as indicated on the **Figure 2(c)**.

### 2.2. Vesicles Preparation

#### 2.2.1. Multilayer Vesicles (MLV)

The phospholipids or sphingolipids in their chloroformic solution were evaporated to a film and resuspended in pure $D_2O$. The liposomes were formed by fast freezing and thawing cycles. The final lipid concentration was 50 mM and MET/lipid molar ratio were fixed to 1:12, 1:10, 1:7, 1:4 M/M.

#### 2.2.2. Small Unilamelar Vesicle (SUV)

Phospholopids in theirs chloroformic solution (10 mg/ml) were lyophylized and resuspended in pure $D_2O$ for a final lipid concentration of 10mM. SUV were formed by 1 Hour bath sonication. For MET containing SUV, the respective chloroformic solutions were mixed before lyophylization.

### 2.3. NMR Experiments

High resolution NMR experiments. Standard 1H-NMR experiments were recorded at 298 K on a Bruker AM-400 spectrometer using a 4000 Hz spectral width, 32 K digitization points, a recycling delay of 2 sec. A presaturation of water resonance was used for all the experiments. Phase sensitive NOESY NMR experiments were recorded at 298 K to detect MET-serine and MET-peptide vicinities (dipolar correlations) with a presaturation of the solvent resonance and mixing times of 250 ms.

Solid-state $^{31}$P-NMR experiments in multilayers (MLV) were performed at 162 MHz. Phosphorus spectra were recorded using a dipolar echo sequence ($\pi/2$-t-$\pi$-t) [40] with a t value of 12 μsec, a $\pi/2$ pulse of 3.8 μs and a broadband two level proton decoupling. Phosphoric acid (85%) was used as external reference. The sample temperature was regulated within 1˚C by a BVT-1000 unit.

Job plots. For all interaction experiments, in a first step, a coarse screening was used by preparing equimolar (5 mM) host/MET mixtures and sample containing excess of MET (9/1, M/M). In the absence of any spectral modification (mainly chemical shift, peak intensity and linewidth), the affinity was considered as negligible and the study was not followed further. Fast exchange kinetics were identified on the spectra by both MET and host chemical shift variations upon MET addition, and the classical method described by Job [41,42] was used to draw the stoechiometry of the complex. Besides, mathematic determination method SIMPLEX (EXPREX, or MURIEL-X) algorithms generously given by Bruno Perly, CEA Saclay, France, for strong complex and in order to draw estimations of the apparent association constant [43].

### 2.4. ESR Experiments: Spin Label Study

Effect of MET on DMPS SUV fluidity was assessed by ESR spin label experiments. Two spin labels (Sigma France) were used: 5-nitroxide stearate (5 NS) and 16-nitroxide stearate (16 NS). This fatty acids self incorporate the SUV and the nitroxide groups provide information of motional freedom of the label in the system. So the former probes the superficial part of the membrane layer, the latter in its hydrophobic core.

The experiments were performed on SUV made with DMPS 10 mg/mL. 1 mg of MET was added in 500 μL SUV solution. 200 μl of the SUV control solution and SUV MET solution were then labelled with 10 μL of spin label solution (5 NS $5 \times 10^{-3}$ M or 16 NS $5 \times 10^{-3}$ M). After 30 min incubation at room temperature, sample was transferred by capillarity in 20 μL Pyrex capillary tube. This tube was placed in a 3 mm diameter quartz holder, and insert into the cavity of the ESR spectrometer.

The ESR spectra were recorded at different controlled temperature (303, 308, 310, 313, 315, 318 and 323 K) with the following conditions: microwave power 20.00

 

mW, modulation frequency 100 kHz, modulation amplitude 2.05 G, receiver gain 6.3 × $10^5$ conversion time 81.92 ms, time constant 81.92 ms. Sweep range was 100 G with a central field value of 3435 G for 5 NS probe, and in the same condition except, modulation amplitude 1.03 G, receiver gain $10^5$ conversion time 40.96 ms, time constant 81.92 ms for 16 NS probe.

The complete membrane incorporation of the spin labels was ascertained by the absence on the spectra of the extremely resolved ESR lines corresponding to free rotating markers.

5 NS experimentations: The values of outer and inner hyperfine splitting were measured ($2T_{//}$ and $2T_{\perp}$ respecttively), on ESR spectra (**Figure 3(b)**), and order parameter S was calculated following the equation [44]:

$$S = 1.723 \times \frac{T_{//} - (T_{\perp} + C)}{T_{//} + 2(T_{\perp} + C)}$$

with

$$C = 1.4 - 0.053 \times (T_{//} - T_{\perp})$$

The increase in the order parameter value means a decrease of local membrane fluidity.

16 NS experimentations: The changes in freedom motion of 16 NS were analysed with the calculation of $\tau_c$, the rotational correlation time. $\tau_c$ was calculated following the formula [45]:

$$Tc = K \times \Delta W_0 \left( \sqrt{(h_o / h_{-1})} - 1 \right)$$

with

$$K = 6.5 \times 10^{-10}\, s \cdot G^{-1}$$

In this formula, $\Delta W_0$ is the peak-to-peak line width of the central line; $h_0$ and $h_{-1}$ are the peak height of the central and high-field lines respectively (**Figure 3(d)**).

The increase in the rotational correlation time means a decrease of local membrane fluidity.

## 3. Results

### 3.1. $^{31}$P-NMR Experiments in MLV

A first attempt for MET affinity and interaction screening was performed by using phospholipid dispersions as membrane model. This model allows to observe the structural and dynamics consequences of the presence of MET at the polar head ($^{31}$P-NMR) by recording NMR spectra under different temperature conditions. Thus the dependence of the interactions with MET following the nature of the polar head group was tested by using different phospholipids, *i.e.* DMPC, sphingomyeline, DMPE, DMPS, DPPG, and also galactocerebrosides and ceramides. At this point, it is noteworthy to recall several basic principles about $^{31}$P-NMR in membranes.

$^{31}$P-NMR chemical shift (the resonance frequency) depends on the orientation of phosphorus nuclei in the field (shielding). The chemical shift difference between the low field and the highfield edges of a $^{31}$P-NMR spectrum is called Chemical Shift Anisotropy (CSA, ppm) and is directly related to the fluidity—reorientation—at the polar head level where the phosphorus nuclei are located. On such spectra a mobile phosphorus group gives a single narrow resonance (several Hz) as detected in true solutions or for small structures (micelles), while phosphorus groups in solid state gives extremely broad contributions (CSA values exceeding 100 ppm). Note that membrane fluidity increases (and CSA decreases) with temperature, with a special jump at the transition temperature between gel phase and liquid crystal structure (e.g. around 297 K for DMPC [46]). Thus the plot of CSA as a function of temperature provides a good overview of membrane dynamics at the polar head level where phosphorus nuclei are located, while the lineshape allows identifying the overall membrane organization (bilayer, hexagonal, isotropic phases). Such plots are presented on the traces of the **Figure 1** (top traces).

The bottom spectrum (**Figure 1**, column A) shows the spectrum of pure DMPC dispersion (MLV), typical of an axia11y symmetric powder pattern, with a chemical shift anisotropy of 65 ppm, classical of a phospholipid (here DMPC) bilayers in their liquid crystalline phase around phase transition (297 K) [47].

As expected for pure DMPC dispersions a CSA decrease (around 18 - 20 ppm) was measured on pure DMPC systems by increasing the temperature (and the membrane fluidity) with the transition-related jump around 297 K [48]. This was also the case for MET containing MLV (MET/DMPC ratios of 1/10 and up to 1/4 M/M, middle and top traces, column A). Also temperature dependence of CSA values was found almost surperimposed on the entire temperature range **Figure 1(c)**). Besides, no significant isotropic contribution typical of detergent effect was observed, thus finally indicating the absence of any interaction with the membrane. Similarly, no interaction with MET was found for cholesterol (up to 20%) containing DMPC dispersions and also for galactocerebrosides, ceramides, and DPPG MLV (not shown). Besides, when DMPE systems were used, only a limited increase in the fluidity (*i.e.* a limited CSA reduction) was observed all over the temperature range, never exceeding 2ppm, even for high MET/DMPE ratios of 1/4.

Such was not the case for the spectra recorded under the same conditions on DMPS (**Figures 1(b)-(d)**) dispersions in the presence of MET. As expected, pure DMPS dispersions gave typical spectra of bilayer structures (see

 

**Figure 1(b)** bottom), with a decrease of the CSA with temperature. The same spectra recorded on MET containing DMPS systems resulted in a reduction of the CSA for MET/DMPS molar ratios exceeding R = 1/10 M/M (trace of the **Figure 1(d)**), while an isotropic contribution appeared for higher R values, with a relative contribution

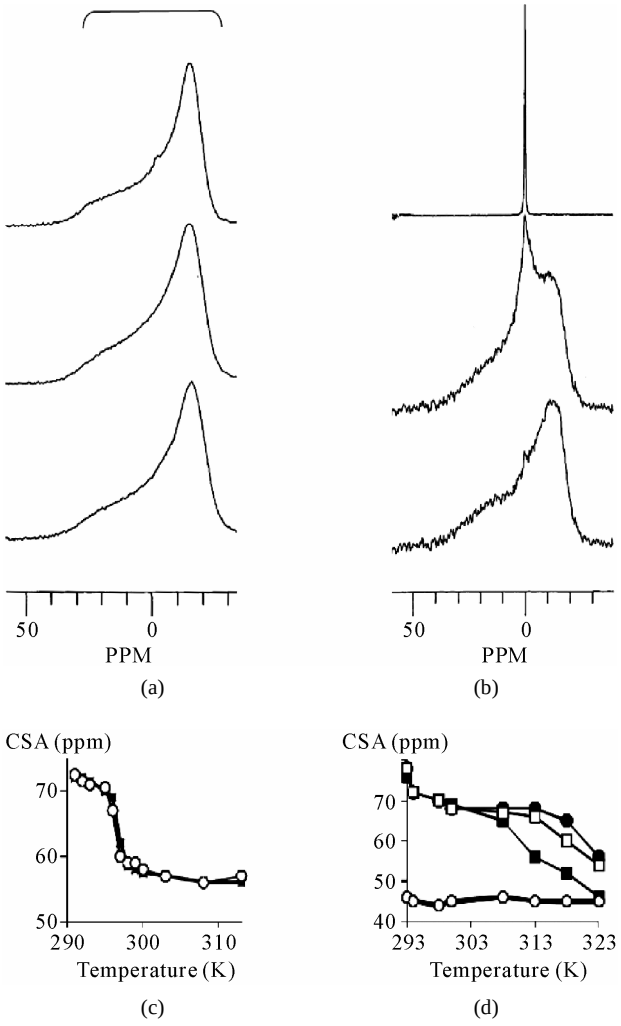


**Figure 1. $^{31}$P-NMR (a) spectra of DMPC dispersion (50 mM, $D_2O$) at 297 K, pure (bottom trace) and in the presence of MET, molar ratios of R = 1/10 and 1/4, M/M, (middle and top traces, respectively); horizontal bracket represent the CSA; (b) spectra of DMPS dispersion (50 mM, $D_2O$) at 313 K, pure (bottom trace) and in the presence of MET, molar ratios of 1/10 and 1/4, M/M, (middle and top traces, respectively; (c) plot of temperature dependence CSA measured on DMPC dispersion spectra of DMPC (○), and in the presence of MET, R = 1/4 M/M (■); (d) plot of temperature dependence CSA measured on DMPS dispersion spectra of DMPS (●), and in the presence of MET, R = 1/10 M/M (□), and R = 1/7 M/M (■); bottom trace (♦) represents the line width of the isotropic contribution observed at R = 1/4 M/M.**

being developed at the expense of the main structure (**Figure 1(b)** middle and top traces). Finally, a single line of less than 50 Hz with was exclusively detected for R = 1/4, indicating that the native structure had been completely replaced by very mobile systems of high fluidity, consistent with a detergent effect or micelle formation.

At this step, the existence of MET/DMPS bilayers appears as highly probable. In order to obtain more precisions, the different building blocks of the phospho- or sphingolipids were considered separately, *i.e.* the polar headgroups and the diacylglycerol backbone. These experiments are the topic of the next section.

## 3.2. $^{1}$H-NMR and ESR of Polar Headgroups and Chains

Headgroups. As both polar groups and MET are soluble in $D_2O$, the first step was achieved by recording spectra on equimolar mixtures and compared to those of pure species of O-phosphocholine (OPC), O-phosphoserin (OPS), O-phosphocolamine(OPCO), and sphingosine. A special care was paid to the aromatic resonances of MET, clearly isolated in the 7 - 9 ppm region (see **Figure 2(a)** for MET structure and nomenclature, bottom trace for the corresponding spectrum of MET).

In the case of OPC, and sphingosine, as no detectable spectrum variation was observed both in chemical shift and linewidth even MET was present in excess (9/1 M/M ratios MET/headgroup) it was assumed that no interacttion occurred and the investigation was not studied further.

OPCO-MET systems only provided minor chemical shift variations that did not allow to propose a precise mode of interaction, stoechiometry or affinity constant (attempts made using Benesi-Hidebrandt method only showed it should not excess 30 $M^{-1}$). The affinity of MET for OPCO was thus considered as very weak.

As significant chemical shift variations were observed on both OPS and MET resonances upon addition of OPS, the continuous variations method was used by recording spectra of different molar ratios MET/OPS, keeping total concentration constant (5 mM, see examples of such spectra on **Figure 2**). This allowed us to use the method to characterize these interactions, as classically described by Job [41].

In such plots, the curves built using concentration weighted chemical shift variations as a function of the molar fraction show a maximum for the molar fraction corresponding to the stoechiometry, here 0.5, which means a 1/1 association (2B). This also allowed the use of numerical simulations (EXPREX2 [43]) that gave an apparent association constant of 3200 $M^{-1}$.

In order to detect spatial vicinities between MET and

 

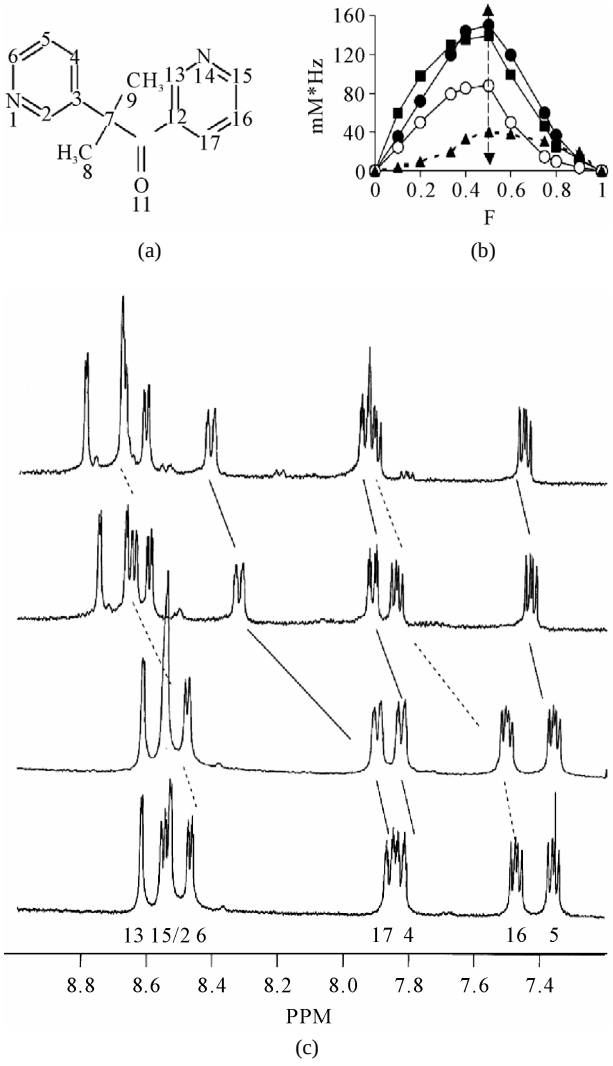


**Figure 2. (a) Metyrapone (MET) formula and proton nomenclature (b) Job-Plots built from concentration-weighted chemical shift variations (mM*Hz) as a function of molar fraction (F), for H5 (7.356 ppm, ■), H4 (7.82 ppm, ●), H16 (7.47 ppm, ○) protons of MET or H2" proton of phosphoserine (4.19 ppm, ▲), 298 K $D_2O$. The total concentrations were kept at 5 mM. Bottom traces with proton numbering below (from bottom to top): aromatic part of the $^1$H-NMR spectra of MET (bottom trace), and in the presence of O-phosphoserine (corresponding molar ratios MET/OPS of 1:0, 9/1; 3/2 and 1/1, respectively.**

OPS, nOesy experiments (mixing time of 250 ms) were then recorded on the 1/1 mixture. Hence weak dipolar correlations were found between one of methylenic protons (4.22 ppm) of OPS and H16, H15 and H13 protons of MET, and also between CH$\alpha$, (4.15 ppm) and H15 not shown). However, molecular dynamics simulations could not lead to propose any stable conformation.

Diacylglycerol backbone. In order to observe chains interaction without any polar head contribution, dispersions of dimyristin and dipalmitin were prepared as for phospholipid MLV. As the $^1$H spectra recorded on the dispersions containing MET (R = 1/1 M/M) were the single sum of the spectra of pure dispersions and MET recorded separately under the same conditions, it was concluded that no interaction occurred at the molecular level (not shown).

### 3.3. ESR Experiments

Spin label experiments were then realised to investigate the membrane fluidity in different temperature conditions at the chain level. Two probes were separately used, 5 NS gate information about superficial membrane fluidity, while 16 NS concerned the inner membrane region. The overall result (**Figures 3(a)** and **(c)**) shows an increase in the mobility of the two probes contribution in the two groups with the temperature increase. As previously described in SUV DMPS model [47,49], the phase transition in control groups occurs near 313 K.

The ESR results were found in full agreement with the experiments recorded by NMR: hence, no interaction with phospholipids and sphingolipids except DMPS was found. In this latter case, an increase in the mobility of the two probes contribution was found when MET was added to DMPS solution.

5 NS results are drawn **Figure 3(a)**. An overhall increase of the membrane fluidity and a shift of the phase transition, 5 degrees lower, could be observed in the MET group.

In the inner compartment, 16NS results (**Figure 3(c)**) show a major effect of MET with a total vanishing of phase transition and a strong increase of the membrane between 303 and 313 K. At the highest temperature (above 315 K) the rotational correlation time of the control group reached the same value as MET group.

However, a true interaction between free serine and MET was very probable. Under a biological point of view, it was important to know if MET could also have interactions with a serine group engaged in a peptide structure. This was tested by using a reference decapeptide NASDSDGQDL as target.

### 3.4. Interactions with the Model Peptide NASDSDGQDL

After purity control and attribution of peptide protons using classical NMR methods [50], the same method as before was used on equimolar mixtures of MET/peptide. By comparison with the spectrum of pure MET (**Figure 4(a)**) significant chemical shift variations were observed on the MET/peptide sample, as shown on **Figure 4(a)**, similar as with OPS/MET preparations. Moreover, the

 

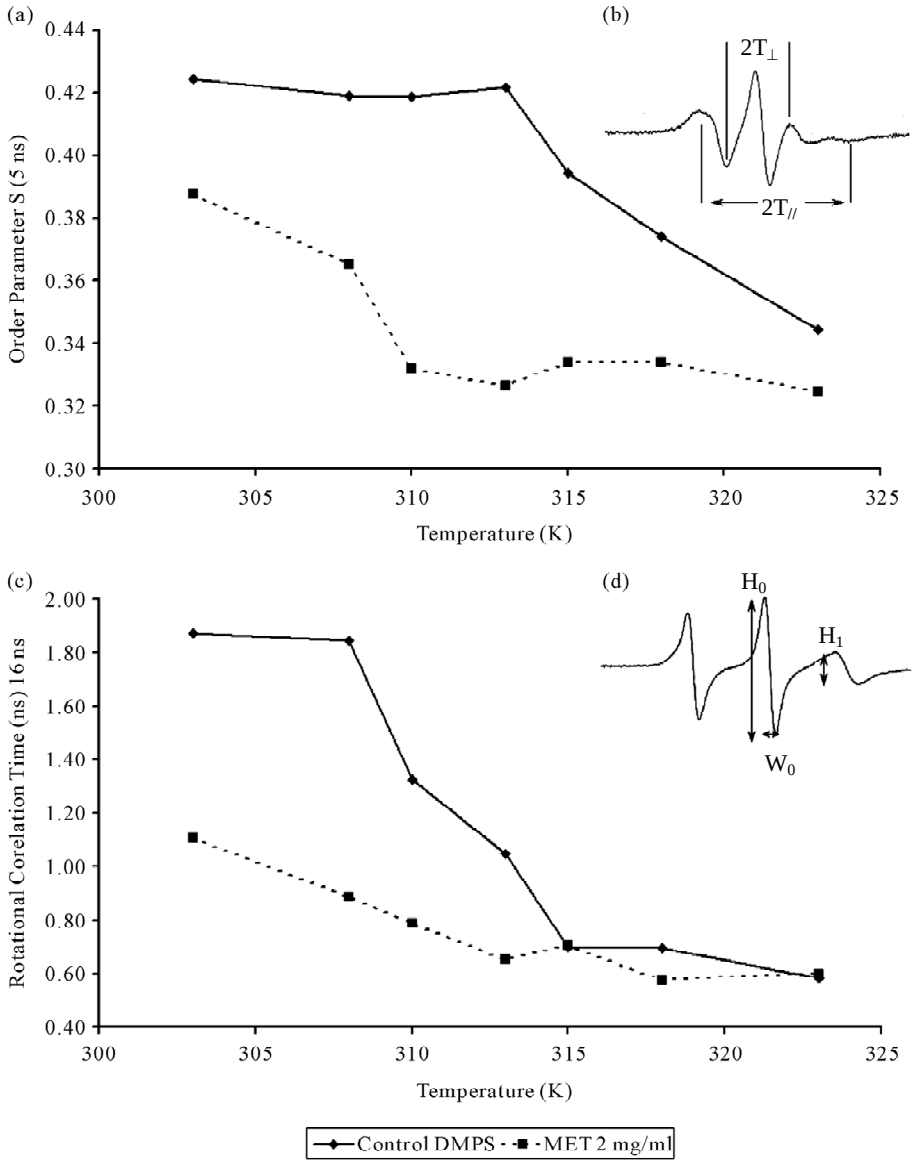


**Figure 3. ESR spin labeling experiment: (a) temperature dependence of the order parameter (5 NS) for pure DMPS (black diamond full line) and in the presence of MET 2 mg/ml (black square, dashed line); (b) typical 5 NS spectrum parameter used for order parameter estimation are inner ($2T_{\perp}$) and outer hyperfine ($2T_{//}$) splitting; (c) temperature dependence of the rotational correlation time (16 NS) for pure DMPC and in the presence of MET 2 mg/ml; (d) typical 16 NS spectrum, parameter used for rotational correlation time was central peak intensity $H_0$, High field peak intensity and the width of the mid-field line $W_0$.**

nOesy experiment then recorded also showed weak dipolar connectivities, mainly between H15, H6 and several peptide protons, suggesting vicinities in the 4 - 6 Å. However, whereas MET well exhibited some vicinities with serine groups, others of more strong intensity were also present with Gly and Gln aminoacids.

## 4. Discussion

The present work primarily investigated the interactions of MET with membrane components, phospholipids, sphingolipids or cholesterol, as evoqued in the literature [51]. The use of multibilayers (dispersions prepared by the freezing-thawing method [52]) combined with solid-state $^{31}$P-NMR spectroscopy allows to investigate both dynamics and structural collective properties of the bilayers. Such a method allows to easily obtain a screening the specificity of interactions with a given phospholipid system. In the present case, as preliminary tests performed

 

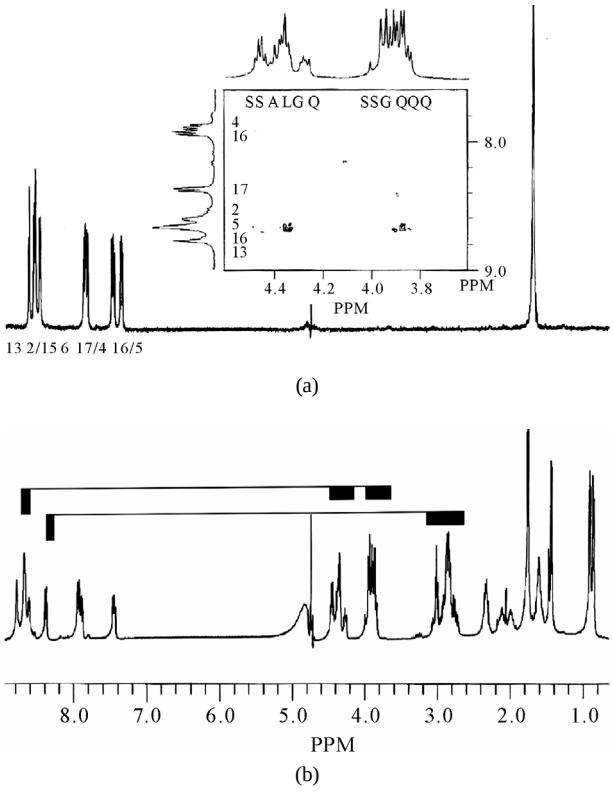


**Figure 4. $^1$H-NMR spectrum of pure MET, 5 mM, 298 K, $D_2O$; (b) same conditions as (a), on a NASDDGQDL/MET preparation (1/1 M/M); Bars indicate dipolar correlations between labelled groups. Inset shows a partial 2D-nOeSY map recorded on the same sample.**

on "standard" lecithin or DMPC small unilamellar vesicles [53], or permeabilization tests on large unilamellar vesicles [54] had given no arguments, an investigation a possible specificity was required. Among the main lipid species tested, MET interactions with DMPS systems were evidenced (from a simple fluidization for low amounts of MET to a drastic detergent effect for molar ratios exceeding 1/7 M/M), and could be precised by studying separately the interactions of MET with lipid building blocks: while the lipidic part seemed not to play any role in the interaction with MET, dramatic spectral modifications were noted in the presence of phosphoserine; moreover, spatial connectivities were identified between aromatic protons of the second cycle of MET (see nomenclature **Figure 2**) and serine groups, even if the fast exchange mode of interaction—that allowed job-plot constructions [55]—could not allow to determine a stable conformation via molecular dynamics calculations. By comparing $^{31}$P-NMR and ESR results, one can observe that MET induces dramatic changes at the polar head level ($^{31}$P), whereas similar liquefying effects were found part of the chain (5 NS), while the same observation could be done at high temperature—at the deep part level of the layer (16 NS). It can thus been proposed that the target for MET interaction is mainly located at the polar head level, were serine groups are present, and that the overall membrane destructuration would result from dynamics perturbation and sterical hindrance, as observed for cholesterol-phospholipid interactions [56]. Nevertheless this interaction with a given amino acid—serine—would also be consistant with the recognition by MET of a signaling site of the target protein guanylate cyclase—a serine containing protein [31]. Thus an attempt was made to test the interaction of MET with a serine containing peptide, *i.e.* to test the consequences for interactions with MET to use a serine engaged in a peptide bound. Despite the presence of true interactions with MET (close to those observed with phosphoserine), and the observation of intermolecular nOes, it was not possible to ascertain any specificity in this case. Especially, dipolar connectivities were also noted with Gly and Gln, suggesting that MET interactions with aminoacids would be at least not specific of serine moieties. However, it must be kept in mind that the peptide used is of small size, hydrophilic and structureless, that means not favorable for any stable conformation or molecular associations found in proteins.

Hence, the future experiments now on course will have to deal with a more realistic model, *i.e.* a "serin-pocket" containing peptide with more stable supramolecular organization.

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