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Comparative optically detected magnetic resonance studies of mammalian phospholipase A_2-lipid interactions

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A major difference between porcine and bovine pancreatic phospholipase A_2 (PA_2) is the relatively low affinity of the bovine enzyme for lipid-water interfaces. We have investigated the binding of porcine, bovine, and equine PA_2 to *n*-hexadecylphosphocholine (C_{16}-PC) micelles using optically detected magnetic resonance (ODMR) spectroscopy. The zero field splittings (ZFS) of the single Trp-3 residue undergo significant changes upon binding of PA_2 to C_{16}-PC micelles. ZFS titrations of PA_2 vs C_{16}-PC indicate that porcine and equine enzymes have similar binding affinity and stoichiometry, while bovine PA_2 binds much more weakly to the lipid-water interfaces. This may be attributed to the differences in the amino acid composition and the conformation of the binding sites for lipid-water interfaces of these enzymes.

ODMR; Phospholipase A_2; Protein-lipid interaction; Tryptophan

1. INTRODUCTION

Mammalian phospholipase A_2, which catalyzes specifically the hydrolysis of the 2-acyl ester linkage in 3-*sn*-phosphoglycerides [1], is secreted by the pancreas as its zymogen which is converted subsequently to the active enzyme by limited proteolysis [2]. The three-dimensional structures of the porcine and bovine enzymes have been determined by X-ray crystallography [3,4]. Although both the zymogen and PA_2 can hydrolyze monomeric phospholipids at a fairly slow rate, only the active enzyme becomes superactivated in the presence of certain organized lipid-water interfaces [5]. Therefore, PA_2 has been proposed to possess a specific binding site for lipid-water interfaces called the interface recognition site. Numerous direct binding studies have been carried out with micellar solutions of nondegradable substrate analogues [5]. These studies have yielded a rather detailed picture of the lipid binding site of the enzyme [6]. The lipid binding domain is situated around the active center and consists mainly of hydrophobic residues. In the porcine enzyme these are Leu-2, Trp-3, Leu-19, Met-20, Leu-31, Leu-58, Leu-64, Val-65, Tyr-69, and Thr-70 [7]. Among these hydrophobic side chains a few positively charged amino acid residues, i.e., Arg and Lys, are in positions that are favorable for interaction with any negatively charged groups that occur in the interface [8].

Although both bovine and porcine PA_2 have comparable enzymatic activities on micellar and monomeric substrates, it is known that the affinity of the bovine enzyme for neutral lipid-water interfaces is much weaker than that of the porcine enzyme [9]. Moreover, bovine PA_2 has a reduced

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Abbreviations: C_{16}-PC, *n*-hexadecylphosphocholine; CMC, critical micellar concentration; ODMR, optically detected magnetic resonance; PA_2, pancreatic phospholipase A_2; ZFS, zero field splittings

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ability to penetrate more densely packed monolayers of lecithins. It is likely that these differences result from changes in the amino acid composition of the binding sites for lipid-water interfaces between the bovine and porcine PA_2. The primary structures of bovine and porcine PA_2 are highly homologous: 85% of the 124 residues are identical [6]. Nevertheless, a loop of 12 residues in these protein structures with only one substitution has been found to have a quite different conformation [7].

In this communication, we report our ODMR measurements of the direct binding of various pancreatic PA_2 enzymes, including porcine, bovine, and equine, to C_{16}-PC micelles. Trp-3 was used as a strategically located intrinsic luminescent probe which lies near the postulated interface recognition site and active site regions [3]. Alteration in the microenvironment of Trp-3 upon binding of PA_2 to C_{16}-PC has been demonstrated by several spectroscopic studies [11–14]. Large ZFS shifts of Trp-3 due to complex formation are obtained with increasing lipid concentrations, which enables us to estimate the complexing stoichiometry. We find that porcine and equine enzymes bind the substrate analogue in a very similar manner, whereas bovine PA_2 has a quite different behavior as reflected by its phosphorescence spectrum and the ZFS titration results. The possible explanations which account for these differences are discussed.

2. MATERIALS AND METHODS

The pancreatic prophospholipase A_2 from pig, horse, and ox were purified from pancreatic tissue and converted into phospholipase A_2 by limited proteolysis as described [2,9,15]. The phospholipid substrate analogue, C_{16}-PC, was synthesized as described by Van Dam-Mieras et al. [11]. Protein concentrations were determined from the absorbance at 280 nm by using $E^{1\%}_{1cm}$ values of 12.3 for bovine and equine PA_2 and a value of 13.0 for the porcine enzyme. All calculations were based on a molecular mass of 13950, 13750, and 13900 Da for porcine, bovine, and equine PA_2, respectively, as can be derived from the amino acid compositions [16–18]. Lipid concentrations were measured by phosphorus determination [19]. Lipid-protein solutions were prepared with final enzyme concentrations of $\sim 10^{-4}$ M. C_{16}-PC concentrations always were well above the CMC of 10^{-5} M. The solvent used was 20% (v/v) glycerol/50 mM aqueous bis-Tris buffer, pH 6, containing 100 mM NaCl and 20 mM $CaCl_2$. Phosphorescence and slow-passage ODMR measurements were made on these solutions after chilling them to 1.2 K using previously described apparatus and procedures [20]. The excitation band centered at 295 nm was selected with a monochromator using 16 nm slits. ODMR measurements were performed by monitoring the tryptophan 0,0-band peak wavelength with 3 nm slits. Microwaves were swept both with increasing and decreasing frequency at the same rate of 30 MHz/s, and the peak ODMR frequencies were averaged to obtain the ZFS parameters $|D|$ and $|E|$.

3. RESULTS

The phosphorescence spectra of porcine, equine, and bovine PA_2 and the complexes formed between these enzymes and C_{16}-PC are quite similar. The 0,0-band wavelengths are listed in table 1. The 0,0-band occurs at 408 nm for both porcine and equine PA_2, and it is found at 407 nm for the bovine enzyme. These 0,0-band wavelength maxima are close to that found for Trp located in an aqueous solvent-exposed environment. Upon complexing of PA_2 with C_{16}-PC micelles, the 0,0-bands of porcine and equine PA_2 undergo only a small shift to 409 nm, although they become significantly narrower, suggesting a decrease in the heterogeneity of the microenvironment upon complex formation. A corresponding decrease in the line width of the 0,0-band upon complex formation was not observed for the bovine PA_2. The spectrum of the complex is essentially the same as that of the enzyme. The effect of complex formation on the $|D| - |E|$ (low frequency) and the $2|E|$ (high frequency) ODMR transitions for these enzymes is shown in fig.1. Substantial shifts in both signals are observed with increasing lipid concentration. The frequency of the $|D| - |E|$ transition is plotted vs the molar ratio of C_{16}-PC to PA_2 in fig.2. $|D| - |E|$ increases continuously with the lipid concentration and levels off with a value of ~ 1.84 GHz at various lipid to protein molar ratios for these enzymes. The ZFS parameter $|E|$ is plotted against the molar ratio of lipid to enzyme in fig.3. $|E|$ decreases rapidly with the lipid concen-

Table 1

Comparison of triplet-state parameters for mammalian PA_2 and complexes formed between PA_2 and C_{16}-PC micelles

	$\lambda_{0\text{-}0}$ (nm)	ν_1 ($\Delta\nu$)[a]	ν_2 ($\Delta\nu$)[a]	D (GHz)	E (GHz)
Porcine PA_2	408.0	1.745(154)	2.448(263)	2.969	1.224
Porcine PA_2 + C_{16}-PC[b]	409.3	1.836(150)	2.268(270)	2.970	1.134
Equine PA_2	407.8	1.758(155)	2.421(253)	2.969	1.211
Equine PA_2 + C_{16}-PC[b]	408.8	1.831(163)	2.288(285)	2.975	1.144
Bovine PA_2	407.0	1.765(165)	2.416(260)	2.973	1.208
Bovine PA_2 + C_{16}-PC[b]	407.0	1.844(173)	2.308(303)	2.998	1.154

[a] Peak ODMR frequencies are in GHz; the values given are the average of frequencies observed by sweeping microwaves in both directions. The numbers in parentheses are linewidths (fwhm) in MHz

[b] Data shown are the average of the values of C_{16}-PC saturated complexes with lipid/protein molar ratio higher than 20, 30, and 80 for porcine, equine, and bovine enzymes, respectively

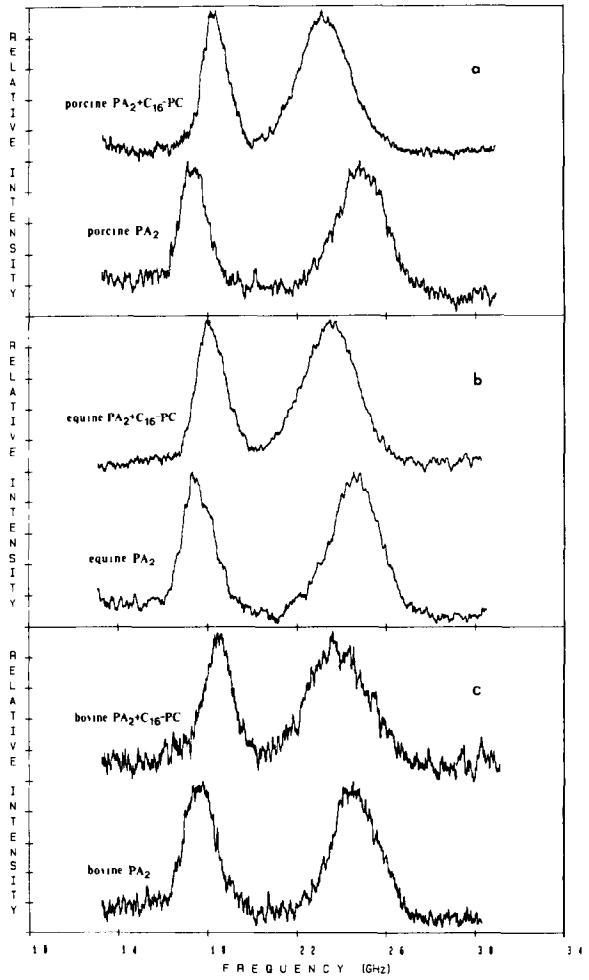

Fig.1. $|D| - |E|$ (low frequency) and $2|E|$ (high frequency) ODMR transitions of (a) porcine, (b) equine, (c) bovine PA_2 and the complexes formed between these enzymes and C_{16}-PC micelles with a molar ratio of 1:100 in each case. The solvent is 20% (v/v) glycerol/50 mM aqueous bis-Tris buffer, pH 6, containing 100 mM NaCl and 20 mM $CaCl_2$. PA_2 concentration is $\sim 10^{-4}$ M. Signal-averaged spectra (~40 repetitions) shown are made with microwaves scanned up in frequency from 1.4 to 3.0 GHz at a rate of 30 MHz/s.

tration for the porcine PA_2 and remains constant at 1.134 GHz when the lipid-protein molar ratio is above 20 (fig.3, circles). The reduction in the $|E|$ value for the equine enzyme (fig.3, triangles) is saturated at a higher lipid/protein molar ratio (~30), attaining a constant value of 1.144 GHz. While the reduction in the $|E|$ parameter for bovine PA_2 with increasing lipid concentration (fig.3, squares) occurs more gradually than that of porcine and equine enzymes, it is found to saturate at a value of 1.154 GHz in the complex. Saturation does not occur until the lipid/protein ratio reaches ~80. A decrease in the value of $|E|$ for each enzyme on binding to C_{16}-PC micelles is consistent with an electric field perturbation, suggesting that the environment of Trp-3 becomes increasingly polar [14]. By contrast, the change in the $|D|$ value with lipid concentration is relatively small for these enzymes. The ZFS data are given in table 1.

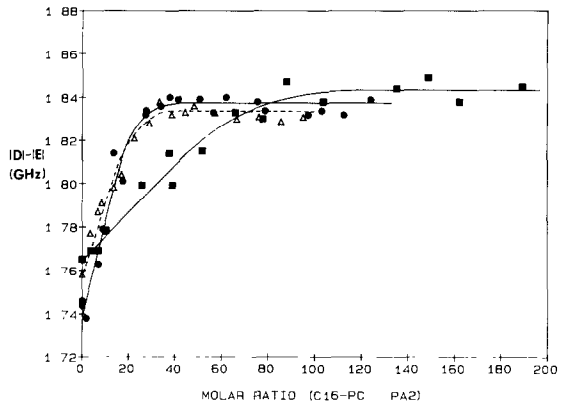

Fig.2. Variation in the $|D| - |E|$ zero field transition frequency of porcine (●), equine (△), and bovine (■) PA_2 upon addition of C_{16}-PC micelles.

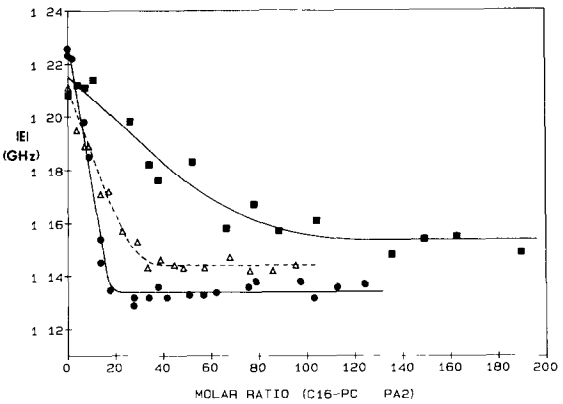

Fig.3. Variation of the ZFS parameter $|E|$ of porcine (●), equine (△), and bovine (■) PA_2 upon addition of C_{16}-PC micelles.

4. DISCUSSION

The results obtained in this study show that Trp-3 of porcine, equine, and bovine PA_2 is involved in the binding of these enzymes to C_{16}-PC micelles. Variation in the local environment of Trp-3 is indicated by the substantial shifts in the ODMR transition frequencies upon complex formation. The $|E|$ parameter is most strongly affected. The ZFS titration data in figs 2 and 3 suggest that the binding constants of these enzymes to the C_{16}-PC micelles have the order: porcine > equine > bovine. These observations are in accord with the results obtained by Dutilh et al. [9], where the affinity of the bovine enzyme for neutral lipid-water interfaces was found to be much weaker than that of the porcine enzyme. Moreover, Meijer et al. [12] found, using UV difference spectroscopy, that the apparent dissociation constants for the complexes formed between pancreatic PA_2 and the C_{16}-PC micelles are 1.8, 5.0, and >13 mM for the porcine, equine, and bovine enzyme, respectively.

It is known from X-ray crystallographic data that clusters of positively charged lysine and arginine residues (e.g., Arg-6, Lys-10, Lys-56, Lys-116, Lys-121, and Lys-122 in porcine enzyme) are present on the enzyme surface [8]. These residues are located around a region containing exposed hydrophobic side chains including the single Trp-3. The active site is located near the center of this lipid binding surface (the interface recognition site). This excess of positively charged residues on the enzyme surface suggests that salt bridge formation with the anionic groups in the micelle could orient the enzyme with respect to the micelle surface. Therefore, variations in the amino acid composition of the interface recognition site among enzymes from different species may affect the binding affinity. Both porcine and equine PA_2 have an arginine residue at position 6, while in the bovine enzyme this is replaced by a neutral asparagine. Substitution of the neutral Asn-6 of bovine PA_2 by the positively charged Arg residue was found to improve not only the lipid binding but also the catalytic activity [22]. However, no decrease in affinity for lipid-water interfaces was observed for Arg-6 → Asn substitution in porcine PA_2 [23]. It was postulated that the difference is due to the fact that the porcine enzyme has a positively charged Lys-10 and His-17 pair in place of the neutral Lys-10, Glu-17 pair which lies very close to Asn-6 in the bovine structure [23]. The side chains of these latter three residues have no net charge and form a 'neutral' cluster in the bovine enzyme. On the other hand, the presence of a positive charge on all three residues, Arg-6, Lys-10, and His-17, in porcine PA_2 may result in a different orientation of these side chains and a stronger complex compared with that of the bovine enzyme. The finding that the Arg-6 → Asn substitution in porcine enzyme has almost no effect on lipid binding is probably due to the fact that sufficient positive charge still remains, main-

taining the porcine-like conformation of these residues [23]. In this respect it is of interest to note that equine PA_2, which has Arg-6, Gln-10, and Lys-17 at these three positions, binds C_{16}-PC micelles with an affinity which lies in between those of the porcine and bovine enzymes. Since there are two positive charges present in this cluster, equine PA_2 should have binding properties more similar to those of the porcine enzyme. In addition to a variation in the N-terminal amino acid composition between the porcine and bovine enzymes, it was found that these enzymes have a different backbone conformation in the structure of the peptide loop 59–70 [3,4,10]. This loop also forms a part of the interface recognition site [8], and thus it may contribute to the differences in binding behavior of these enzymes.

In this study, we have shown, using ODMR, that Trp-3 is affected by the binding of pancreatic PA_2 from pig, horse, and ox to C_{16}-PC micelles. Changes in ZFS are consistent with the introduction of electric fields from charged groups in the vicinity of the tryptophan site. This conclusion, based on ODMR, that the Trp environment becomes more polar when PA_2 binds to the micelle, is in marked contrast to the interpretation based on fluorescence titrations [11]. In the latter, the enhanced fluorescence quantum yield and blue shift upon complex formation with *n*-dodecylphosphocholine micelles has been explained as a hydrophobic perturbation. By inspection of the Trp location in a space filling model of the enzyme, on the other hand, it is difficult to imagine that the indole ring could reach the hydrophobic core of the micelle. It seems more logical that it is surrounded by the large polar head groups. A possible reason for this inconsistency could involve differences in the physical state of the sample, i.e., that its fluorescence is studied at ambient temperature in a fluid medium, whereas its ODMR is measured at cryogenic temperatures in a rigid medium. Thus diffusional processes are possible during the excited state lifetime in the ambient temperature fluorescence experiments. Through diffusional processes, the enzyme may reach a conformation in which Trp in its excited state is stabilized in a hydrophobic site. On the other hand, the lowest energy conformation(s) with respect to ground electronic state Trp is frozen out for the ODMR measurements, in which Trp remains located in the region of the polar head groups. Titration of the enzymes with C_{16}-PC monitoring ZFS changes shows that the binding affinity decreases in the order, pig > horse > ox, in agreement with earlier measurements using other methods. These measurements appear to correlate with changes which occur in the net positive charge on residues 6, 10, and 17 located in a cluster on the surface of the enzyme. A decrease in net positive charge gives rise to a reduced binding affinity to C_{16}-PC micelles.

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