Investigation of phospholipase-lipid interactions by optical detection of triplet state magnetic resonance spectroscopy

Su-Yau Mao, August H. Maki* and Gerard H. de Haas[+]

Department of Chemistry, University of California, Davis, CA 95616, USA and [+]*Biochemisch Laboratorium, Rijksuniversiteit Utrecht, Padualaan 8, 3508 TB Utrecht, The Netherlands*

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We have investigated the binding of porcine pancreatic phospholipase A_2 (PA_2) to *n*-hexadecylphosphocholine ($C_{16}PN$) micelles using optical detection of triplet state magnetic resonance (ODMR) spectroscopy. The zero field splittings (zfs) of the single Trp^3 residue undergo significant changes upon binding of PA_2 to $C_{16}PN$ micelles. Zfs titrations of PA_2 vs $C_{16}PN$ indicate that the binding stoichiometry is $C_{16}PN:PA_2$ $\sim$ 25. A reduction of the $|E|$ parameter from 1.227 to 1.135 GHz is postulated to result from Stark effects caused by the binding of a polar group (possibly phosphocholine) near Trp^3 in the PA_2-$C_{16}PN$ micelle complex.

ODMR *Phospholipase A_2* *Protein-lipid interaction* *Tryptophan*

1. INTRODUCTION

Mammalian phospholipase A_2 (PA_2), which catalyzes specifically the hydrolysis of the ester bond at the C2 position of 3-*sn*-phosphoglycerides [1], is secreted by the pancreas as its zymogen which is converted subsequently to the active enzyme by limited proteolysis [2]. Whereas the zymogen can degrade only monomeric substrate molecules, the active enzyme is also able to bind to and to digest efficiently organized phospholipid structures such as micelles and liposomes [3]. It has been proposed [3] that a particular surface region of PA_2, called the interface recognition site (IRS), interacts specifically with certain lipid-water interfaces with a concomitant optimization of the active site architecture. The N-terminal region of the enzyme is thought to play an important role in the recognition process. Although progress has been made in identifying amino acid residues involved in the binding of PA_2 to lipid-water interfaces [3–6], and information regarding the binding stoichiometry to micellar substrate analogs has been obtained, the nature of the interaction forces is not yet clearly understood.

The luminescence of aromatic amino acids in proteins is known to be influenced by interactions with the local environment [7,8]. The single tryptophan residue of PA_2, which occurs at position 3 (Trp^3) can serve as a strategically located intrinsic luminescent probe which lies near the postulated IRS and active site regions [9]. Furthermore, it is apparently involved directly in the interaction of pancreatic phospholipases with lipid-water interfaces [4,10,11].

Here, we present the first application of optical detection of triplet state magnetic resonance (ODMR) spectroscopy to the investigation of protein-lipid interactions. Although ODMR spectroscopy of Trp residues in proteins has been car-

* To whom correspondence should be addressed

Abbreviations: $C_{12}PN$, *n*-dodecylphosphocholine; $C_{16}PN$, *n*-hexadecylphosphocholine; CMC, critical micellar concentration; IRS, interface recognition site, ODMR, optical detection of triplet state magnetic resonance; PA_2, porcine pancreatic phospholipase A_2; photo-CIDNP, photochemically induced dynamic nuclear polarization; zfs, zero field splittings

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ried out for more than a decade [12,13], the sample generally has been dissolved in a cryosolvent such as glycerol-buffer or ethylene glycol-buffer, even if the protein is found naturally associated with membranes. ODMR spectroscopy probes the local environment of aromatic amino acid residues, of which Trp has been the most extensively studied. The zero field splittings (zfs) are sensitive to local electric fields (Stark shifts) [14,15] and thus can serve to monitor changes which occur in the environment. We report ODMR measurements on porcine PA_2 as the enzyme is bound to *n*-hexadecylphosphocholine ($C_{16}PN$) micelles. Large zfs shifts which occur upon complex formation with micelles enable us to estimate the complexing stoichiometry. We find that changes in the Trp zfs are consistent with the location of Trp[3] in a polar region, near charged residues.

For purposes of comparison, the porcine PA_2 zymogen was also investigated, as was the interaction of active enzyme and zymogen with *n*-dodecylphosphocholine ($C_{12}PN$).

2. MATERIALS AND METHODS

Porcine pancreatic prophospholipase A_2 was purified from pancreatic tissue and converted into PA_2 by limited proteolysis as described previously [2]. The phospholipid substrate analogs, $C_{12}PN$ and $C_{16}PN$, were synthesized as described by Van Dam-Mieras et al. [4]. Protein concentrations were determined from the absorbance at 280 nm using $E^{1\%}_{1\,cm}$ = 13.0 [4]. Lipid concentrations were measured by phosphorous determination [16]. Lipid-protein solutions were prepared with final enzyme concentrations of approx. 10^{-4} M. $C_{16}PN$ concentrations were always well above the CMC of 10^{-5} M, while $C_{12}PN$ solutions were prepared below its CMC of 1 mM. Phosphorescence and slow passage ODMR measurements were made on these solutions using previously described apparatus and procedures after chilling them to 1.2 K [17,18]. The sample was excited with a 100 W high pressure Hg arc equipped with a 12 cm path length aqueous $NiSO_4$ (500 g/l) infrared filter and a Corning CS 7-54 glass filter. The excitation band centered at 295 nm was selected with a monochromator using 16 nm slits. The phosphorescence 0,0-band was monitored at its peak wavelength using a monochromator with 3 nm slits for ODMR measurements. Microwaves were swept both with increasing and with decreasing frequency at the same rate of <34 MHz/s, and the peak ODMR frequencies were averaged to obtain $|D|$ and $|E|$.

3. RESULTS

The phosphorescence of porcine PA_2 when excited at 295 nm is structured, and characteristic of Trp. The 0,0-band occurs at 408 nm. Upon complexing of PA_2 with $C_{16}PN$ micelles, the 0,0-band undergoes only a small shift to 409 nm, although it becomes significantly narrower, suggesting a decrease in the heterogeneity of the microenvironment upon complex formation. The effect of complex formation on the $|D| - |E|$ and the $2|E|$ ODMR signals of PA_2 is seen in fig.1. The frequency of the $|D| - |E|$ transition is plotted vs the molar ratio of $C_{16}PN$ to PA_2 in fig.2. The zfs parameters $|E|$ and $|D|$ are plotted against the

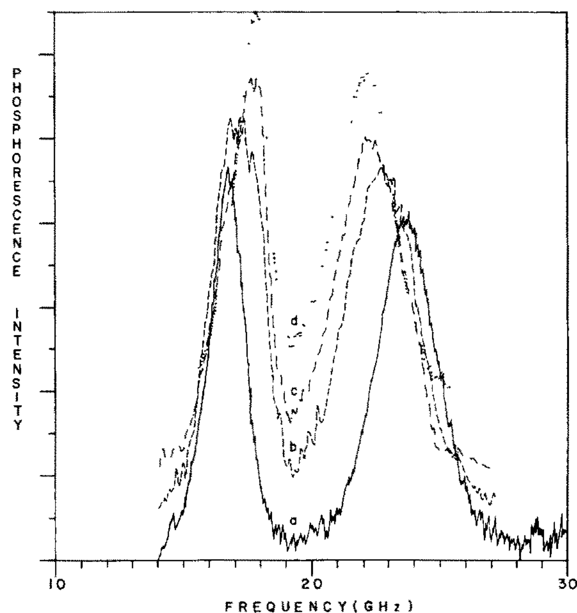

Fig.1. $|D| - |E|$ (low frequency) and $2|E|$ (high frequency) ODMR transitions of porcine PA_2 titrated with $C_{16}PN$ micelles. The molar ratio $C_{16}PN:PA_2$ = (a) 0, (b) 4.6, (c) 13.8, and (d) 37.6. The solvent is 20% (v/v) glycerol/aqueous 50 mM Bistris buffer, pH 6, containing 100 mM NaCl and 20 mM $CaCl_2$. Signal-averaged spectra (40 repetitions) shown are made with microwaves scanned down in frequency from 2.5 to 1.4 GHz at a rate of 34 MHz/s.

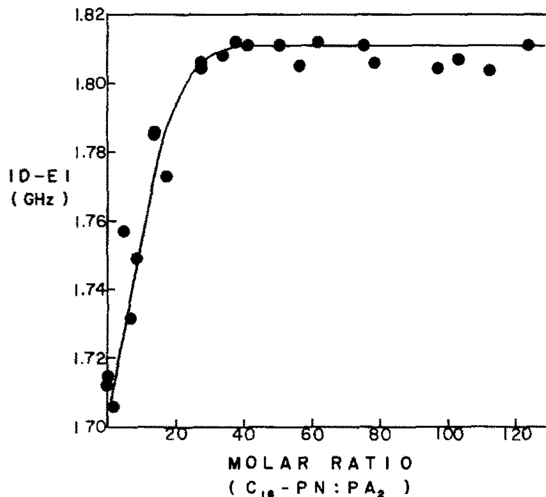

Fig.2. Variation in the $|D| - |E|$ zero field transition frequency of PA_2 upon addition of $C_{16}PN$ micelles. The samples are prepared in the solution described in fig.1.

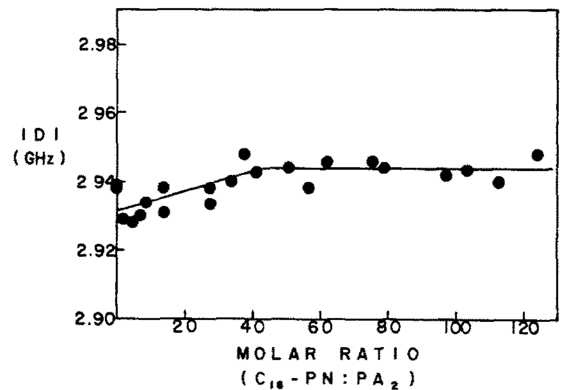

Fig.4. Variation of the zfs parameter $|D|$ of PA_2 upon addition of $C_{16}PN$ micelles.

molar ratio of lipid to enzyme in figs 3 and 4, respectively.

The phosphorescence spectra of porcine PA_2 and its zymogen have similar 0,0-band wavelength maxima, but the spectrum of the zymogen is somewhat better resolved. The zfs of the zymogen, $|D| = 2.97$, $|E| = 1.25$ GHz, are similar to those of PA_2, and near those found for Trp exposed to aqueous solvent [19]. Only relatively small zfs shifts occur for the zymogen upon addition of $C_{16}PN$ micelles at a concentration which results in complete binding of the active enzyme. $|E|$ is reduced from 1.25 to 1.219 GHz, a much smaller effect than occurs on binding PA_2 to $C_{16}PN$ micelles (figs 2,3). Upon addition of a 4-fold molar excess of $C_{12}PN$ (~0.68 mM) to the zymogen, no changes in the zfs or ODMR linewidths are observed. When $C_{12}PN$ is added to PA_2 under the same conditions, however, $|E|$ is reduced from 1.227 to 1.199 GHz, and the linewidths of the $|D| - |E|$ and $2|E|$ transitions increase by 35% to 235 and 365 MHz, respectively.

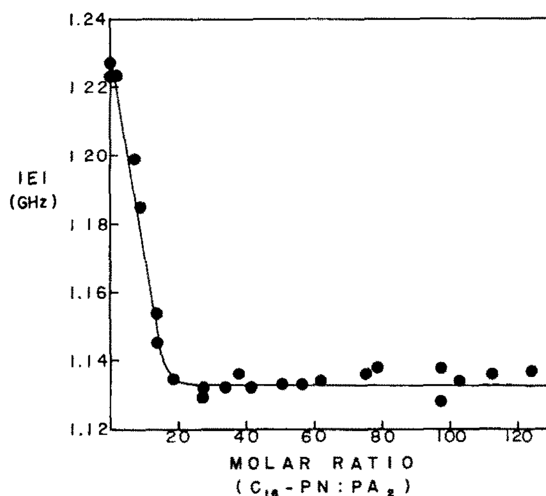

Fig.3. Variation of the zfs parameter $|E|$ of PA_2 upon addition of $C_{16}PN$ micelles.

4. DISCUSSION

The results presented in section 3 show that Trp^3 of porcine PA_2 is involved in the binding of the enzyme to $C_{16}PN$ micelles. Variation in the local environment is reflected mainly in the triplet excited state zfs in which the $|E|$ parameter is most strongly affected. The Trp^3 environment of the zymogen, on the other hand, shows only relatively small perturbations upon addition of micelles. These observations are in accord with those made previously on the fluorescence [4] and photo-CIDNP [10,11] of Trp^3. It is found that in PA_2, Trp^3 interacts strongly with the lipid-water interface in the enzyme-micelle complex, while no such interaction is found for the zymogen. Although the $C_{12}PN$ concentration was kept below the room temperature CMC, we cannot rule out the possibility that micelles form in the cryosolvent at

reduced temperature. Our results suggest that micelles are present, however, since PA_2 selectively undergoes zfs shifts and broadening of the ODMR lines similar to those found upon addition of $C_{16}PN$ at the same molar ratio.

An estimate of the binding stoichiometry may be obtained from the data in figs 2 and 3. The zfs changes saturate after the addition of about a 25-fold molar excess of lipid, which suggests that the stoichiometry of enzyme-lipid complexing is approx. 1:25. Previous work on this system [3] using isothermal calorimetry, light scattering, and equilibrium gel filtration concluded that in binding enzyme molecules the micelles, originally containing about 155 monomers, form smaller 80-monomer units, each complexed with two enzyme molecules. The difference between our results and the previous ones which predict a 1:40 binding stoichiometry may well be due to the different conditions for preparation of the complexes. In the present measurements, the samples are chilled to a temperature at which the 20% glycerol cryosolvent becomes rigid ($\sim -8°C$); the structure of the enzyme-micelle complexes could well change during chilling in a manner which results in an altered lipid-poor stoichiometry.

Finally, the nature of the local environment of Trp^3 in the PA_2-micelle complex is suggested by the phosphorescence and ODMR data. The 0,0-band wavelength of 409 nm compares well with that found for Trp in an aqueous solvent-exposed environment, rather than a hydrophobic environment, where a significantly more red-shifted origin is found [12,13,19]. The $|E|$ parameter of Trp is found to be large in a hydrophobic environment – larger than is found for a solvent-exposed environment. For example, the $|E|$ value for the tripeptide Gly-Trp-Gly in 50% (v/v) ethylene glycol-water (a typically solvent-exposed site) is 1.234 GHz [20], not far from that of PA_2 (fig.3). Trps which are buried in hydrophobic regions are represented by azurin B, $|E|$ = 1.394 GHz [20,21], and α-chymotrypsin, $|E|$ = 1.349 GHz [17]. One of the smallest values of $|E|$ found thus far, 1.159 GHz, occurs in the C subunit of protein kinase [20], and is not far from the value found for the PA_2-$C_{16}PN$ micelle complex in this work (fig.3). It has been suggested [20] that the low $|E|$ value in the C subunit is caused by a phosphorylated threonine adjacent to the Trp. The ODMR results presented here suggest that Trp^3 of PA_2 is located in a polar region in the enzyme-micelle complex; it possibly is subjected to the electric field of the phosphocholine head group.

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