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Optically Detected Magnetic Resonance Studies of Porcine Pancreatic Phospholipase A_2 Binding to a Negatively Charged Substrate Analogue†

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ABSTRACT: The direct binding of porcine pancreatic phospholipase A_2 and its zymogen to 1,2-bis(heptanylcarbamoyl)-*rac*-glycerol 3-sulfate was studied by optical detection of triplet-state magnetic resonance spectroscopy in zero applied magnetic field. The zero-field splittings of the single Trp^3 residue undergo significant changes upon binding of phospholipase A_2 to lipid. Shifts in zero-field splittings, characterized mainly by a reduction of the *E* parameter from 1.215 to 1.144 GHz, point to large changes in the Trp^3 local environment which accompany the complexing of phospholipase A_2 with lipid. This may be attributed to Stark effects caused by the binding of a charged group near Trp^3 in the enzyme–lipid complex. The cofactor, Ca^{2+}, which is strongly bound to the enzyme active site, has an influence on the bonding, as reflected by smaller zero-field splitting shifts. A relatively small change in the Trp environment was observed for the interaction of the zymogen with lipid.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of the 2-acyl ester linkage in 3-*sn*-phosphoglycerides. The pancreatic enzymes have an absolute requirement for Ca^{2+} ions, which bind to the active site (Dijkstra et al., 1981a). In mammals PA_2[1] is secreted by the pancreas as its zymogen, $proPA_2$, which is converted subsequently to the active enzyme by trypsin, resulting in the loss of a heptapeptide from the N-terminal part (de Haas et al., 1968b). Although both PA_2 and $proPA_2$ are able to catalyze the hydrolysis of molecularly dispersed substrates at a fairly slow rate, only the active enzyme becomes superactivated in the presence of certain organized lipid–water interfaces (Volwerk & de Haas, 1982). Regardless of the various hypotheses, no generally accepted explanation has been given to date for the extremely effective heterogeneous catalysis. A particular surface region of PA_2 has been postulated as an interface recognition site, which is distinct from the active site but is important nonetheless for binding of the protein to the organized lipid substrate.

In order to identify the amino acid side chains of PA_2 involved in the recognition of, and interaction with, lipid–water interfaces, numerous direct binding studies have been carried out with micellar solutions of nondegradable substrate analogues (Volwerk & deHaas, 1982). Mostly neutral phosphocholine-containing lipids have been employed in order to

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; C_{16}-PN, *n*-hexadecylphosphocholine; cmc, critical micellar concentration; diC_7-dicarbamoyl-GS, 1,2-bis(heptanylcarbamoyl)-*rac*-glycerol 3-sulfate sodium salt; D-diC_7GS, 2,3-diheptanoyl-*sn*-glycerol 1-sulfate sodium salt; D-diC_8GS, 2,3-dioctanoyl-*sn*-glycerol 1-sulfate sodium salt; EDTA, ethylenediaminetetraacetic acid; ODMR, optical detection of triplet-state magnetic resonance; PA_2, porcine pancreatic phospholipase A_2; $proPA_2$, porcine pancreatic prophospholipase A_2; SDS, sodium *n*-dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; zfs, zero-field splitting(s).

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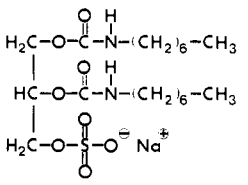

FIGURE 1: Structural formula of diC_7-dicarbamoyl-GS.

allow the presence of Ca^{2+} ions which do not perturb the lipid organization. These studies have yielded a rather detailed picture of the lipid binding site of the enzyme (Verheij et al., 1981). 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} (Slotboom et al., 1982). 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 (Dijkstra et al., 1981b). This appears to be in line with observations that the pancreatic enzymes interact preferentially with anionic phospholipids (de Haas et al., 1968a; Bonsen et al., 1972). Direct binding and kinetic studies have been performed by using single-chain negatively charged detergents (Hille et al., 1983a,b), where the induced aggregation and activation of PA_2 were shown to occur at submicellar detergent concentrations. The maximal specific activities were rather low, however, as was known already from earlier studies (Bonsen et al., 1972); PA_2 shows low activities toward single-chain substrates. It became of interest to study the enzyme activity on double-chain anionic substrates, which are more nearly like the naturally occurring substrates for PA_2. While the kinetic study on diacyl-α-glycerol sulfates is in progress (Hille, 1983), the direct binding of a double-chain anionic substrate analogue to PA_2 and $proPA_2$ is investigated in the present study. The fatty acyl ester bonds are replaced by carbamoyl ester linkages (Figure 1), which are known to be fully resistant to PA_2 hydrolysis (Gupta & Bali, 1981).

In this paper, we report our ODMR measurements of porcine PA_2 as the enzyme is bound to diC_7-dicarbamoyl-GS. Specific effects of Ca^{2+} on the binding also are investigated. Large zfs shifts of Trp^3 due to complex formation are obtained with increasing lipid concentrations. Furthermore, the changes in the Trp zfs suggest that Trp^3 becomes located in a polar region. $ProPA_2$ also is studied for purposes of comparison, as is the effect of high Ca^{2+} concentrations on the Trp^3 microenvironment in PA_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 (Nieuwenhuizen et al., 1974). The negatively charged substrate analogue diC_7-dicarbamoyl-GS was synthesized as described by Hille (1983). Sodium *n*-dodecyl sulfate (SDS) was obtained from Kodak. Protein concentrations were determined from the absorbance at 280 nm with $E^{1\%}_{1\,cm} = 12.3$ and 13.0 for zymogen and active enzyme, respectively. Lipid concentrations were determined by weight. Lipid–protein solutions were prepared with final enzyme concentratioins of ca. 10^{-4} M. The buffers used at pH 6.0 were 20% (v/v) glycerol/aqueous 10 mM Bis-Tris, containing either 10 mM $CaCl_2$ or 2 mM EDTA. At pH 8.0, 20% (v/v) glycerol/aqueous 10 mM Tris was used.

Phosphorescence and slow-passage ODMR measurements were made on these solutions after chilling them to 1.2 K by using previously described apparatus and procedures (Maki & Co, 1976; Ghosh et al., 1984). The excitation band centered at 295 nm was selected with a monochromator using 16-nm slits. The phosphorescence was collected at right angles in a 600 groove/mm 1-m monochromator (McPherson Model 2051) and was detected with a cooled (EMI Model 9789QA) photomultiplier tube. ODMR measurements were performed by monitoring the Trp 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 $|E|$. The swept frequencies were calibrated by a microwave frequency counter (Hewlett-Packard Model 5351A).

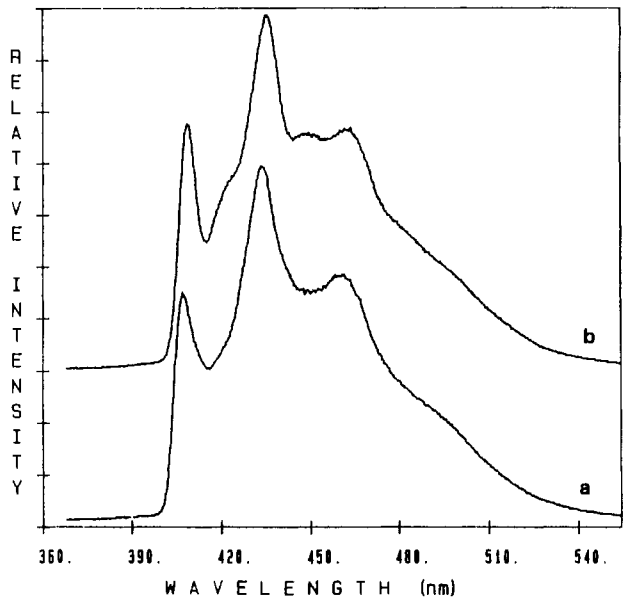

FIGURE 2: Phosphorescence spectra at 4.2 K of (a) porcine PA_2 and (b) PA_2 + diC_7-dicarbamoyl-GS (1.6 mM). The solvent is 20% (v/v) glycerol/aqueous 10 mM Bis-Tris buffer, pH 6.0, containing 2 mM EDTA. PA_2 concentration is ca. 10^{-4} M.

Results

At pH 6.0, in the absence of Ca^{2+}, the phosphorescence spectrum of porcine PA_2 when excited at 295 nm is characteristic of Trp. The 0,0-band occurs at 407 nm, near that found for Trp exposed to aqueous solvent (Hershberger et al., 1980). Upon addition of the substrate analogue diC_7-dicarbamoyl-GS, the 0,0-band undergoes only a small shift to 409 nm and becomes better resolved (Figure 2), suggesting a more homogeneous microenvironment of Trp^3 upon complex formation. The effect of complex formation on the $|D| - |E|$ amd $2|E|$ ODMR transitions of PA_2 is shown in Figure 3. Substantial shifts in both signals are observed with increasing lipid concentration. The frequency of the $|D| - |E|$ transition is plotted vs. the lipid concentration in Figure 4, curve a. $|D| - |E|$ increases continuously with the lipid concentration up to ca. 1.6 mM. At higher concentrations, it remains constant at about 1.820 GHz, which is 80 MHz larger than that of the free enzyme. The zfs parameter $|E|$ is plotted against the lipid concentration in Figure 5a. $|E|$ falls rapidly with increasing lipid concentratioin up to ca. 1.6 mM and levels off with a constant value of 1.144 GHz, which is 80 MHz smaller than that of PA_2. By contrast, there is no apparent variation in $|D|$ upon addition of lipid. Both the $|D| - |E|$ and the $2|E|$ ODMR lines are broadened in the presence of diC_7-dicarbamoyl-GS. For comparison, Table I lists the zfs parameters for PA_2 binding to both diC_7-dicarbamoyl-GS micelles and C_{16}-PN micelles (Mao et al., 1985).

Table I: Comparison of Triplet-State Parameters for PA_2 and Complexes Formed between PA_2 and Various Micelles

	$\lambda_{0,0}$ (nm)	ν_1 ($\Delta\nu$)[a]	ν_2 ($\Delta\nu$)[a]	D (GHz)	E (GHz)
PA_2[b]	407.6	1.746 (154)	2.445 (263)	2.968	1.223
PA_2 + C_{16}-PN[b]	409.0	1.834 (148)	2.263 (271)	2.966	1.132
PA_2 (2 mM EDTA)	407.2	1.741 (158)	2.430 (247)	2.956	1.215
PA_2 + diC_7-dicarbamoyl-GS (2 mM EDTA)[c]	408.6	1.820 (182)	2.289 (353)	2.964	1.144
PA_2 (10 mM Ca^{2+})	408.0	1.747 (145)	2.429 (246)	2.961	1.214
PA_2 + diC_7-dicarbamoyl-GS (10 mM Ca^{2+})[d]	408.8	1.803 (159)	2.322 (321)	2.964	1.161

[a] Peak ODMR frequencies are in GHz; the values given are the average of frequencies observed by sweeping in both directions. The numbers in parentheses are line widths (fwhm) in MHz. [b] Data from Mao et al. (1985). The lipid–protein molar ratio was 102.81:1. A systemetic error of about 30 MHz in the $|D| - |E|$ transition frequency (ν_1) has been corrected in the table. [c] The lipid concentration was 6.4 mM. [d] The lipid concentration was 3.2 mM.

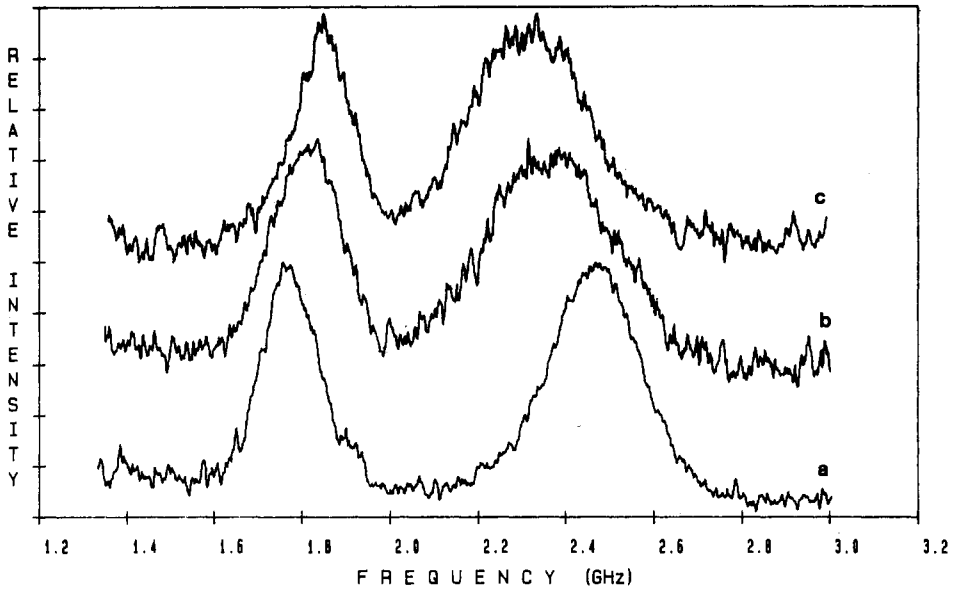

FIGURE 3: $|D| - |E|$ (low-frequency) and $2|E|$ (high-frequency) ODMR transitions of porcine PA_2 titrated with diC_7-dicarbamoyl-GS. The lipid concentrations are (a) 0, (b) 0.8, and (c) 1.6 mM. The samples are prepared in the solution described in the legend of Figure 1. Signal-averaged spectra (ca. 50 repetitions) shown are made with microwaves scanned up in frequency from 1.4 to 3.0 GHz at a rate of 30 MHz/s.

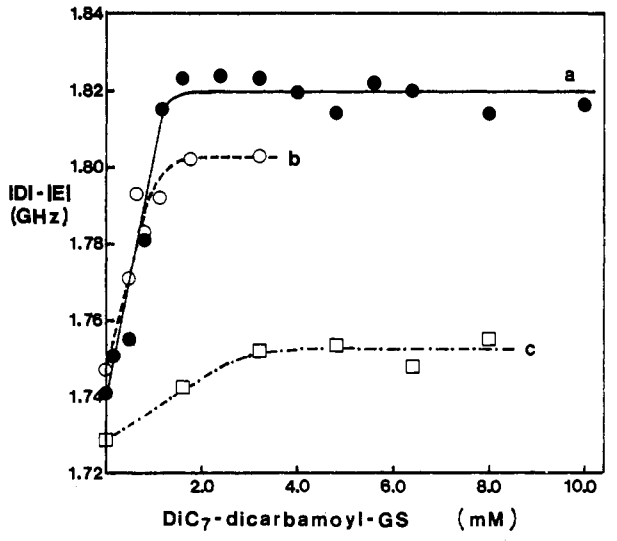

FIGURE 4: Variation in the $|D| - |E|$ zero-field transition frequency of porcine PA_2 and $proPA_2$ upon addition of diC_7-dicarbamoyl-GS. The solvent is 20% (v/v) glycerol/aqueous 10 mM Bis-Tris buffer, pH 6.0; phospholipase concentration is ca. 10^{-4} M. (a) PA_2 + 2 mM EDTA (●); (b) PA_2 + 10 mM $CaCl_2$ (○); (c) $proPA_2$ + 2 mM EDTA (□).

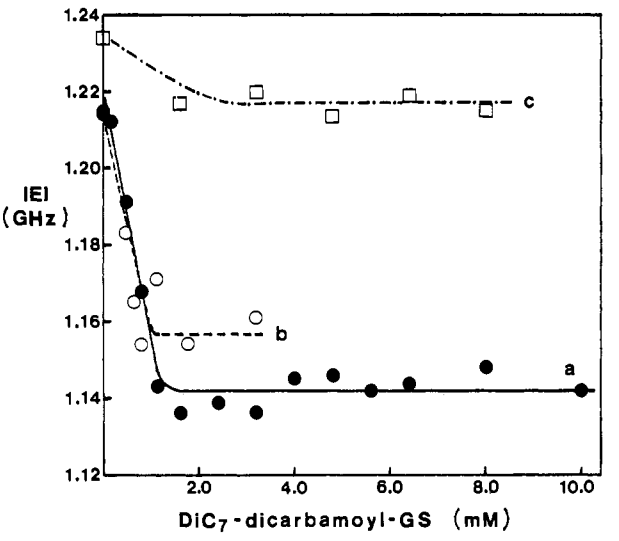

FIGURE 5: Variation of the zfs parameter $|E|$ of PA_2 and $proPA_2$ upon addition of diC_7-dicarbamoyl-GS: (a) PA_2 + 2 mM EDTA (●); (b) PA_2 + 10 mM $CaCl_2$ (○); (c) $proPA_2$ + 2 mM EDTA (□).

The possible specific effect of Ca^{2+} ions on PA_2–diC_7-dicarbamoyl-GS binding was also studied. In the presence of 10 mM Ca^{2+}, the phosphorescence spectrum of PA_2 is similar to that of PA_2 without Ca^{2+}, and upon complexation of PA_2 with lipid, the 0,0-band also shifts to 409 nm and becomes narrower. Titration of PA_2 with lipid, however, revealed some effect of Ca^{2+} on the $|D| - |E|$ and $2|E|$ transitions. Smaller zfs shifts induced by addition of lipid occur for PA_2 when Ca^{2+} ions are bound to the active site. The results also are compared with those in the absence of Ca^{2+} in Table I. In the presence of Ca^{2+}, $|D| - |E|$ increases from 1.747 to only 1.803 GHz, while $|E|$ only undergoes reduction from 1.214 to 1.161 GHz (Figures 4 and 5, curves b). Again, as in the absence of Ca^{2+}, no change was observed for $|D|$. The $|D| - |E|$ and $2|E|$ ODMR

Table II: ODMR Frequencies and zfs Parameters of Porcine Phospholipase A_2

enzyme	pH	origin (nm)	ν_1 ($\Delta\nu$)[a]	ν_2 ($\Delta\nu$)[a]	D (GHz)	E (GHz)
PA_2 (2 mM EDTA)	6.0	407.2	1.741 (158)	2.430 (247)	2.956	1.215
PA_2 (10 mM Ca^{2+})	6.0	408.0	1.747 (145)	2.429 (246)	2.961	1.214
PA_2 (100 mM Ca^{2+})	6.0	407.8	1.749 (163)	2.420 (236)	2.959	1.210
PA_2 (255 mM Ca^{2+})	6.0	408.0	1.754 (155)	2.412 (237)	2.960	1.206
PA_2 (2 mM EDTA)	8.0	407.4	1.743 (160)	2.428 (256)	2.957	1.214
PA_2 (10 mM Ca^{2+})	8.0	407.8	1.757 (151)	2.424 (271)	2.969	1.212
PA_2 (100 mM Ca^{2+}	8.0	408.2	1.753 (158)	2.412 (237)	2.959	1.206
PA_2 (255 mM Ca^{2+})	8.0	408.6	1.750 (152)	2.416 (260)	2.958	1.208

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

lines also are broadened upon binding to lipid, although to a smaller extent.

The zymogen of pancreatic PA_2 does not bind to organized, zwitterionic lipid–water interfaces, probably because of its distorted lipid binding domain (Volwerk & de Haas, 1982), although it interacts with interfaces having a net negative surface charge (Hille et al., 1983a; Volwerk et al., 1984). It was, therefore, of interest to study the interaction of this protein with both double-chain and single-chain anionic substrate analogues. $proPA_2$ has a somewhat better resolved phosphorescence spectrum than does PA_2, with a 0,0-band maximum at 409 nm. The zfs of the zymogen, $|D| = 2.962$ GHz and $|E| = 1.234$ GHz, are similar to those of PA_2. Only relatively small changes in the zfs or ODMR line widths were observed, however, for the zymogen upon the addition of diC_7-dicarbamoyl-GS up to 8.0 mM (Figure 4 and 5, curves c), a concentration that results in complete binding of the active enzyme. Binding of $proPA_2$ to the single-chain detergent molecule SDS induces nearly the same changes in the zfs of the tryptophan: $|D|$ remains constant and $|E|$ shifts to 1.216 GHz at concentrations of SDS up to 13.5 mM.

Apart from having a function in enzymatic catalysis, Ca^{2+} ions also play a role in the binding of phospholipases to lipids. At alkaline pH, PA_2 is known to bind a second Ca^{2+} near the N-terminal region to improve the affinity of the enzyme for lipid–water interfaces (Donné-Op den Kelder et al., 1983). In order to gain some further insight on a possible effect of Ca^{2+} on the Trp^3 microenvironment, solutions of PA_2 in buffers containing high Ca^{2+} ion concentrations (100 or 255 mM) at pH 6.0 and 8.0 were prepared for ODMR measurements. The optical and ODMR data are listed in Table II. The small changes in phosphorescence spectra and ODMR signals are nearly within the experimental precision, demonstrating that the binding of Ca^{2+} to the N-terminal region of PA_2 does not strongly perturb Trp^3.

Discussion

The direct binding of pancreatic PA_2 with zwitterionic, single-chain *n*-alkylphosphocholine analogues has been studied by a number of techniques, including UV difference spectroscopy, fluorescence spectroscopy, Sephadex equilibrium filtration, and microcalorimetry (Volwerk & de Haas, 1982). More recently, the binding has been studied by ODMR (Mao et al., 1985). When the lipid concentration is below the cmc of the substrate analogue, both PA_2 and $proPA_2$ bind only one lipid monomer per protein molecule in the active site, and the microenvironment of Trp^3 is not directly affected. In the presence of zwitterionic detergent micelles only PA_2 forms a lipid–protein complex consisting of 2–3 proteins and 60–90 detergent monomers per micelle. Binding of PA_2 to these micelles is a rapid, exothermic process with hydrophobic interactions stabilizing the lipid–protein complex (Soares de Araujo et al., 1979). Alteration in the microenvironment of Trp^3 has been demonstrated by several spectroscopic studies (van Dam-Mieras et al., 1975; Egmond et al., 1980, 1983; Mao et al., 1985). The zymogen does not recognize these micellar interfaces, and no "higher molecular weight" complexes are formed.

The pancreatic phospholipases are known to hydrolyze anionic phospholipids more completely than zwitterionic phospholipids (de Haas et al., 1968a). Furthermore, this preference has been confirmed recently by kinetic studies on negatively charged monolayer systems (Hendrickson et al., 1981). Moreover, the enzyme has several positively charged amino acid side chains among the residues comprising the lipid binding domain, suggesting the influence of salt-bridge formation with negatively charged groups in the micelle which would thus orient the enzyme with respect to the micelle surface (Dijkstra et al., 1981b). Therefore, interactions between charged groups might play an important role in the interfacial binding and catalytic activity of PA_2. Studies on single-chain anionic substrate analogues containing a sulfate group as the polar head (Hille et al., 1983a,b) revealed that porcine PA_2 forms a detergent–protein aggregate in a two-step process. High-affinity binding of only two detergent molecules to the lipid binding domain of the enzyme occurs at a concentration far below the cmc, followed by the formation of high molecular weight lipid–protein complexes consisting of about 6 enzyme molecules and 40 sulfate monomers. Strong perturbation of Trp in PA_2 which accompanies the first binding step was observed. Although the zymogen of porcine PA_2 seems to form comparable high molecular weight aggregates with these detergents, these complexes are different spectroscopically from those formed by PA_2, and higher detergent concentrations are required for their formation. In these "pseudomicellar" complexes only PA_2 but not the zymogen becomes superactivated. A related observation is that of Volwerk et al. (1984), who found that titration of PA_2 with SDS-containing C_{16}-PN micelles leads to a large (ca. 200%) increase in fluorescence, whereas only a relatively small (less than 50%) fluorescence enhancement is found by using $proPA_2$. There is, apparently, a smaller change in Trp environment in $proPA_2$ binding than when the active enzyme binds to micelles.

Returning to our studies on the double-chain anionic substrate analogue, diC_7-dicarbamoyl-GS, at pH 6.0 in the absence of Ca^{2+}, the results described show that Trp^3 of porcine PA_2 is affected by the binding of the enzyme to the negatively charged glycerol sulfate. Perturbation of the Trp^3 local environment upon the binding of lipid is reflected in the triplet excited state zfs in which the parameter $|E|$ is greatly reduced. The zfs changes saturate after the addition of about 1.6 mM lipid, suggesting that the molar ratio of the lipid–enzyme complex is ca. 16:1. Our zfs titrations of PA_2 vs. diC_7-dicarbamoyl-GS reveal no evidence for the two-step binding process described by Hille et al. (1983a). Previous work on diC_7-dicarbamoyl-GS, D-diC_7GS, and D-diC_8GS (the latter

two lipids are D isomers of diacyl-α-glycerol sulfate, which are resistant to PA_2 hydrolysis) using equilibrium gel filtration concluded that in the absence of Ca^{2+} lipid–protein complexes are formed by both PA_2 and $proPA_2$ at a concentration less than one-tenth of the cmc. These have a "molecular weight" of ca. 70 000, and a lipid–protein molar ratio of 2 is reached in the first step of binding. Our results, however, show that the lipid–protein aggregation which occurs at submicellar concentration does not perturb the microenvironment of Trp^3; this does not occur until a higher lipid concentration is reached. Our results imply that the amino acid residues that constitute the so-called "hot" sites for high-affinity binding of two double-chain sulfate monomers may not be in the vicinity of Trp^3.

As to the nature of the local environment of Trp^3 in PA_2 complexes with diC_7-dicarbamoyl-GS, both phosphorescence spectra and ODMR data suggest that Trp^3 is located in a polar region. Previous ODMR studies of PA_2 binding to the zwitterionic detergent micelles C_{16}-PN (Mao et al., 1985) show that a large reduction in the zfs $|E|$ parameter of Trp accompanies complex formation. The complex has one of the smallest values of $|E|$ found thus far, 1.132 GHz (Table I), whereas $|D| = 2.966$ GHz is very close to that of the free enzyme. it was concluded 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. In the present study, the Trp zfs in the complex formed between PA_2 and the double-chain sulfate lipid micelles, $|D| = 2.964$ GHz and $|E| = 1.144$ GHz, indicate that the extent of the perturbation of Trp^3 is slightly less than that which accompanies PA_2 complexing with C_{16}-PN micelles. Possible explanations for the difference in zfs shifts are the following: (a) the electric fields generated by the phosphocholine head group may well be different from those originating from the sulfate group, or (b) the distances between these charged groups and Trp^3 in the complexes may be somewhat different. The effects of environment on the zfs and ODMR line widths of Trp are likely the result of Stark mixing of T_1 with other excited triplet states (van Egmond et al., 1975). Interactions with electrically charged groups will thereby perturb the electron distribution of Trp, directly affecting the spin–spin dipolar interaction that is primarily responsible for the zfs. Electric fields originating from charged atoms located in the indole plane can induce mixing of T_1 with T_n ($n > 1$) states also having $^3(\pi,\pi^*)$ configurations. Such Stark mixing would perturb primarily the value of $|E|$ (Maki, 1984).

It is of interest that when $proPA_2$ was titrated with either single-chain or double-chain anionic substrate analogues, only a relatively small perturbation of the Trp environment was observed from the phosphorescence spectra and the ODMR transition frequencies. This agrees with the results obtained by Hille et al. (1983a) and Volwerk et al. (1984); the zymogen of porcine PA_2 interacts with anionic or negatively charged mixed detergent micelles, but the extent of the perturbation of the single Trp environment is less than that found for the active enzyme.

PA_2 has an absolute requirement for Ca^{2+} in the hydrolysis of any type of substrate. The pH–V_{max} profile of porcine PA_2 shows a sharp optimum at pH 6 at low Ca^{2+} concentrations, while a plateau between pH 6 and pH 9 was found for high Ca^{2+} concentrations (50–100 mM) (Volwerk & de Haas, 1982). The influence of Ca^{2+} on the interfacial binding process was confirmed recently by Donné-Op den Kelder et al. (1981, 1983). At alkaline pH, porcine PA_2 is known to bind two Ca^{2+} ions per protein molecule. One Ca^{2+} is strongly bound to the active site and is essential for enzyme activity. A second Ca^{2+} binds more weakly to the protein and restores the micelle binding capacity of the enzyme at high pH values. Glu^{71} has been identified as one of the ligands for Ca^{2+} in the low-affinity site (Donné-Op den Kelder et al., 1983). Because of deprotonation of Glu^{71} at alkaline pH, binding of PA_2 to micelles requires Ca^{2+} to neutralize the negative charge of the carboxylate group. Slotboom et al. (1978) showed that binding of Ca^{2+} to the low-affinity site of porcine PA_2 perturbs the microenvironment of Trp^3 and shifts the pK of the α-NH_3 group from 8.4 to 9.3. They concluded that the second Ca^{2+} ion binds close to the N-terminal part. However, no great perturbation of the Trp^3 environment by high Ca^{2+} concentrations is observed in the present study either at pH 6.0 or at pH 8.0. According to the X-ray model (Dijkstra et al., 1981a; Donné-Op den Kelder et al., 1983) the distance between the proposed second Ca^{2+} binding site and the Trp^3 seems to be too large for a direct charge effect on the zfs.

Finally, for zwitterionic substrates, it was concluded from the initial velocity patterns obtained for porcine PA_2 and short-chain lecithin that the addition of substrate and Ca^{2+} to the enzyme is random, and the interactions show a synergistic effect; that is, the affinity of the enzyme–Ca^{2+} complex for the interfacial substrate is higher than that of the free enzyme, and Ca^{2+} is bound more strongly to the enzyme–micelle complex than to the free enzyme (Volwerk & de Haas 1982). For single-chain anionic substrate analogues, Hille et al. (1983a) showed that Ca^{2+} does not affect the first binding step involving high-affinity binding of two detergent monomers to the protein surface. Ca^{2+}, however, seems to increase the aggregation of monomeric protein. From the results described in the present study it is clear that Ca^{2+} also has an influence on the binding of PA_2 to the double-chain sulfate lipids. In the presence of Ca^{2+}, the complex formation also perturbs the Trp^3 microenvironment as reflected in the zfs parameters, $|D| = 2.964$ GHz and $|E| = 1.161$ GHz (Table I), although to a smaller extent relative to the perturbation caused by binding in the absence of Ca^{2+}. At 10 mM Ca^{2+}, only the active site is saturated by Ca^{2+} at pH 6 ($K_{Ca} = 2.3$ mM at pH 6; Pieterson et al., 1974), while the low-affinity site is not occupied ($K_{Ca} \simeq 50$ mM at pH 6; Slotboom et al., 1978); hence this effect probably is due to the different orientation of the substrate molecules caused by Ca^{2+} ion in the active site. In addition, Hille (1983) found that, in the presence of Ca^{2+}, lipid–protein complexes form with a much higher molecular weight and probably with a different composition. Such differences in structure may be the cause of the Ca^{2+} effect on the ODMR properties of Trp^3 that we observe in our measurements.

Registry No. C_{16}-PN, 93597-88-7; PA_2, 9001-84-7; diC_7-dicarbamoyl-GS, 101653-96-7; $proPA_2$, 37350-21-3; L-tryptophan, 73-22-3.

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Protoheme–Carbon Monoxide Geminate Kinetics†

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ABSTRACT: Recombination kinetics of CO to protoheme after laser photolysis have been measured vs. temperature and viscosity. A 25-ns laser pulse was focused on the sample to produce an excitation rate of 10^9/s per heme. This temporarily populates the heme–CO state of dissociated pairs which either separate or recombine on a picosecond time scale in viscous glycerol–water solutions. From the equilibrium amplitude of the fraction dissociated during the laser pulse, the geminate recombination rate constant is calculated to be 3×10^9/s. This rate coefficient is only weakly dependent on temperature or viscosity. As previously observed the fraction that escapes depends on the solvent viscosity [Marden, M. C. (1983) Ph.D. Thesis, University of Illinois–Urbana]. A model consisting of a single barrier plus diffusive escape is used to simulate the kinetics during and just after the flash.

The kinetics of carbon monoxide binding to protoheme has been extensively studied on a microsecond time scale. After photodissociation of CO from protoheme, a single bimolecular process has been observed and characterized as a function of temperature and viscosity (Alberding et al., 1976; Hasinoff, 1977; Marden, 1980). The observed amplitude of this process decreases as the solvent viscosity increases. This implies either that the photosensitivity decreases or that a fast geminate process exists which accounts for the missing fraction of the change in absorbance. A geminate phase has been seen for protoheme in frozen solvents when there is no escape of the ligand; values for the rate in the liquid phase have been extrapolated from below 100 K as 10^9/s (Alberding et al., 1976) and 26×10^9/s (Doster et al., 1982). Geminate kinetics have been observed in heme proteins on a nanosecond/microsecond scale (Austin et al., 1975; Duddell et al., 1980; Lindqvist et al., 1981) and later extended to the picosecond time scale (Chernoff et al., 1980; Friedman et al., 1985).

Model compounds have been prepared that can approximate the oxygen binding equilibrium curves observed in proteins. This implies that the ratio of the on/off rates is correct; a more

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