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The environment of tryptophan in pig pancreatic phospholipase A_2 bound to bilayers

Mahendra Kumar Jain [a] and Badri P. Maliwal [b]

[a] *Department of Chemistry, University of Delaware, Newark, DE 19716, and* [b] *Department of Biological Chemistry, University of Maryland Medical School, Baltimore, MD 21201 (U.S.A.)*

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Binding of pig pancreatic phospholipase A_2 to ternary codispersions of diacylphosphatidylcholine/lysophosphatidylcholine/fatty acid (100:22:22, mole ratio) is monitored by the increase in intrinsic fluorescence intensity of the single tryptophan residue. The fluorescence is quenched by the brominated fatty acid components in the ternary codispersions. The quenching efficiency is in the order: 11,12-dibromo- > 9,10-dibromo- > 6,7-dibromo- > 2-bromo fatty acid. The quenching efficiency of the 9,10-brominated derivatives of the three components in the ternary codispersions is in the order diacylphosphatidylcholine > fatty acid > lysophosphatidylcholine. Two isomers of diacylphosphatidylcholine with 9,10-dibromo substituents on chain 1 or 2 are equally efficient quenchers. While succinimide also quenches the fluorescence of the free and the membrane bound enzyme, the tryptophan residue in both systems is not accessible to 1-methylnicotinamide. These results are rationalized by a hypothesis that the acyl chains of the substrate interacts with the tryptophan residue of pig pancreatic phospholipase A_2, which is readily accessible to water soluble neutral quenchers both in the free and the bound state.

Introduction

Binding of pig pancreatic phospholipase A_2 to substrate interface [1] is accompanied by a change in the spectral properties of the single tryptophan residue in the 3-position [2–5]. In the three-dimensional structure of this enzyme the tryptophan residue is fairly well separated from the catalytic site [6]. Therefore, it is generally believed that the tryptophan is part of the interfacial recognition site (distinct from the catalytic active site) which is involved in the binding of the enzyme to the substrate interface. In order to understand the nature of the interaction of phospholipase A_2 with the substrate interface, we have studied the fluorescence properties of the enzyme bound to phospholipid bilayers. These studies demonstrate a coupling between the binding of the substrate to the catalytic site and the perturbation of the tryptophan residue at the interfacial recognition site.

Materials and Methods

Phospholipase A_2 was provided by Professor G.H. de Haas. All lipids used in this study were purchased from Avanti (Birmingham), Medmark (Munich), or Nu-Check Prep (Elysian, MN). A sample of 1-octadec-9-enyl, 2-hexadecyl-phosphatidylcholine was provided by Professor R. Bittman. Octyltryptophan and 1-methylnicotinamide were from Sigma, and 2-bromopalmitic acid from Aldrich. All other brominated lipids were prepared by reaction of the corresponding unsaturated lipids with bromine in carbon tetrachloride. The prod-

ucts were judged homogeneous by thin-layer chromatography and proton NMR spectra.

All fluorescence measurements were made with an SLM-4800 spectrofluorimeter interfaced to a microcomputer. Excitation was at 295 nm (slit 2 nM) and emission scanned from 300 nm to 400 nm (slits 2 nm) in ratio mode. All data reported here is derived from uncorrected spectra.

Fluorescence lifetimes were measured by the phase-shift method [8] at 30 MHz, with 2,5-diphenyl-1,3,4-oxadiazole ($\tau = 1.2$ ns) and terphenyl ($\tau = 1.05$ ns) in ethanol as life-time references [9].

Sonicated lipid dispersions were prepared from the dry film of premixed lipids dispersed in aqueous buffer by a bath type sonicator (Sonicor).

Binding studies were done with the ternary codispersions containing dialkylphosphatidylcholine/1-palmitoyllysophosphatidylcholine/palmitic acid (100 : 22 : 22, mole ratio). For kinetic studies the ternary codispersions contained diacylphosphatidylcholine.

Lipid titrations shown in Fig. 2 and 3 were done by adding the aqueous codispersions of the appropriate lipid mixture to the fluorophore (8 μM) in the cuvette. For these measurements excitation was set at 295 nm and emission at 336 nm with slit widths at 4/4 nm.

For quenching studies with 1-methylnicotinamide and succinimide excitation was set at 300 nm in order to eliminate inner filter effects. The soluble quenchers were added in aliquots directly to the curvette, and the brominated lipids were always added as ternary codispersions. All Stern-Volmer plots in the case of succinimide were either linear or curved upwards suggesting presence of static quenching. In the case of upward curvature of quenching plots, results were analyzed for both collisional (K_d) and static (K_s) quenching constants [11].

Results

On binding of pig pancreatic phospholipase A_2 to micellar [2] and bilayer forms [4] of nonhydrolyzable substrate analogs there is an increase in the fluorescence quantum yield and a blue shift of the emission maximum from 341 nm to 333 nm. It should be pointed out that in bilayers these changes are seen only with the ternary codispersions. No noticeable change in the fluorescence intensity is observed in the presence of the vesicles of pure ditetradecylphosphatidylcholine or codispersions of any two of the three premixed lipids under various conditions including at the mid-point of gel to liquid-crystalline phase transition temperature range. With the ternary codispersions containing dialkylphosphatidylcholine/ 1-acyllysophosphatidylcholine/fatty acid (10 : 2 : 2, mole ratio), the emission maximum and half-height width of the emission band decreases, and the quantum yield increases, which suggests the presence of the tryptophan fluorophore in a more hydrophobic environment when the enzyme is bound to the lipid interface [2–5].

As outlined elsewhere [4], from the dependence of the fluorescence change upon the lipid concentration, we obtained the apparent dissociation constant (nK_d) for the bound enzyme. Such values for several lipid systems are summarized in Table I, which also contains K_m and V_{max} values, that is the kinetic Michaelis-Menten constants obtained from the measurement of the initial zero-order rates of hydrolysis as a function of the substrate concentration in the ternary codispersions containing diacylphosphatidylcholine. The apparent dissociation constants and the kinetic constant K_m are approximately equal for a variety of lipid dispersions for which such measurements could be made. This indicates that the introduction of bromine in the fatty acid or the acyl chains does not significantly affect the binding of phospholipase A_2 to ternary codispersions as manifested in the K_m values. Similarly, the V_{max} values do not change significantly by the various bromine substitutions, thus suggesting that such structural modifications do not have a significant effect on the catalytic turnover.

The catalytic action of phospholipase A_2 on the ternary codispersions containing the various brominated components suggest that the quality of interface in these dispersions is essentially identical. However, some of the brominated dispersions do not induce a significant increase in the fluorescence of the enzyme. This is probably due to the quenching of the tryptophan fluorescence by bromo substituents. The fluorescence quenching effect on incorporation of phospholipase A_2 into ternary codispersions containing brominated lipids

TABLE I

THE BINDING AND KINETIC CONSTANTS FOR PHOSPHOLIPASE A_2 (30°C)

The acyl-chain lengths of the three components in the ternary codispersions are given. I_{rel} is relative intensity compared to that of *N*-acetyltryptophanamide. τ is the phase life-time measured at 30 MHz.

Lipid (PC + LPC + FA)	nK_d (μM)	K_m (μM)	V_{max} (I.U.)	I_{rel}	τp
14 + 16 + 16	270	240	460	0.50	1.89
14 + 16 + 2-Br-16	152	200	350	0.54	2.1
14 + 16 + 6,7-diBr-18	120	150	230	0.37	2.2
14 + 16 + 9,10-diBr-18	–	130	270	0.28	1.86
14 + 16 + 11,12-diBr-18	–	200	300	0.29	1.79
14 + 9,10-diBr-18 + 16	200	240	280	0.42	1.39
1-(9,10-diBr)-18, 2-16 + 16 + 16	–	210	280	0.17	1.42
2-(9,10-diBr)-18, 1-16 + 16 + 16	–	210	280	0.17	1.42
None	–	–		0.30	1.9

is shown in Figs. 1 and 2. The change in fluorescence intensity of phospholipase A_2 in the ternary dispersions depends upon the position of the bromo substituent in the acyl chain. The 9,10-dibromo and 11,12-dibromo fatty acids are more effective quenchers than the 6,7-dibromo or 2-bromo fatty acids. Similarly, the quenching efficiency for the 9,10-dibromo-substituted components in the ternary codispersions is in the order: diacylphosphatidylcholine > fatty acid > lysophosphatidylcholine. Quenching by diacylphosphatidylcholine isomers is essentially identical whether the 9,10-dibromo substitution is on 1-acyl or on

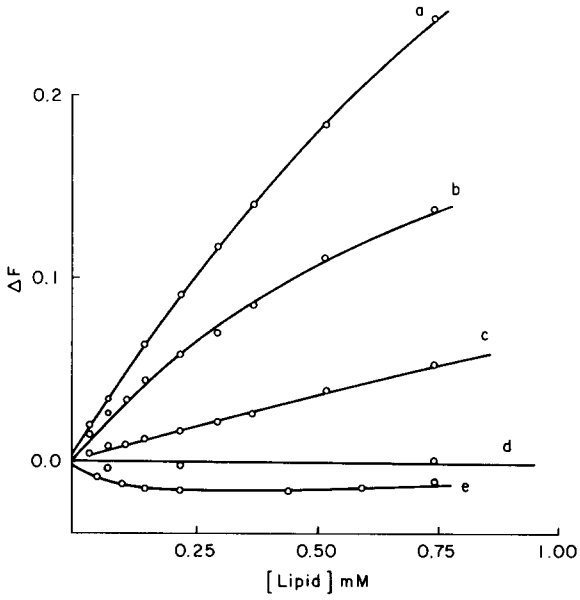

Fig. 1. Change in the fluorescence intensity of pig pancreatic phospholipase A_2 to the ternary codispersions containing ditetradecylphosphatidylcholine/1-palmitoyllysophosphatidylcholine/fatty acids (100:22:22, mole ratio): a, 2-bromopalmitic acid; b, palmitic acid; c, 6,7-dibromostearic acid; d, 9,10-dibromostearic acid; e, 11,12-dibromostearic acid. Enzyme 8 μM, pH 8.0, temperature 30°C.

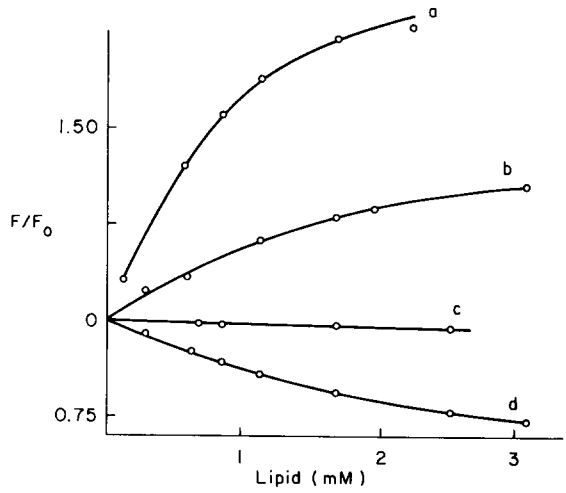

Fig. 2. Change in the fluorescence intensity of pig pancreatic phospholipase A_2 to ternary codispersions: a, none of the components is brominated; b, with 9,10-dibromostearoyllysophosphatidylcholine; c, 9,10-dibromostearic acid; d, 1-hexadecyl-2-(9,10-dibromooctadecyl)phosphatidylcholine. Other conditions as in Fig. 1.

2-acyl chains (data not given in Table I). These results therefore suggest that position and the structure of the bromo substituent determines the quenching efficiency. By assuming that incorporation of the probe does not perturb the orientation and the lateral distribution of the components of the bilayer, in a simplistic way the quenching by brominated derivatives, with bromine localized at different positions of the fatty acid chain, could be used as a 'dip-stick' type of measure of the relative degree of penetration of the tryptophan in the hydrophobic region of the bilayer. For example, the octyl ester of tryptophan is maximally quenched by 2- and 6-bromo fatty acids (Fig. 3), and the three 9,10-dibromo components in the ternary codispersions are equally effective quenchers of this tryptophan (data not shown). According to this criteria the tryptophan of pig-pancreatic phospholipase A_2 should penetrate to the depth of C_9–C_{11} from the carboxyl group. However, this conclusion for phospholipase A_2 is not consistent with the observation that the quenching efficiency of the three components of the ternary codispersions, in which the bromo substituents are in 9,10-positions, is considerably different for the tryptophan of phospholipase A_2 (cf. Fig. 2), and also with the fact that this residue is readily accessible to succinimide (see below).

Table II shows the results of succinimide- and *N*-methylnicotinamide-mediated quenching experi-

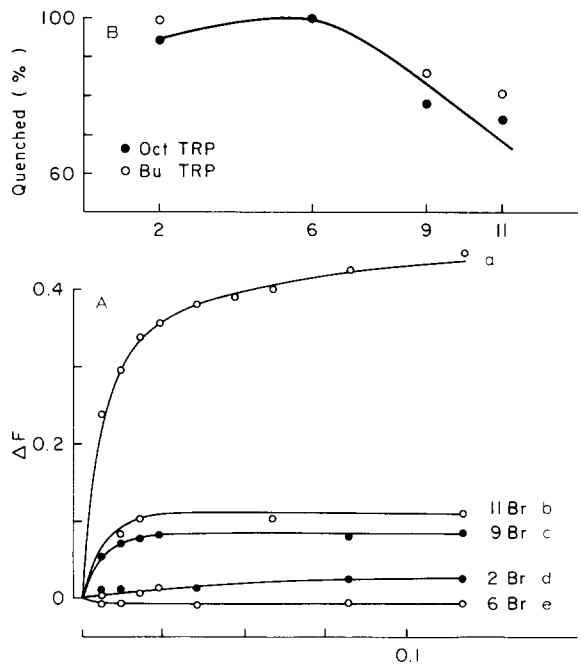

Fig. 3. (A) Change in the fluorescence intensity of the octyl ester of tryptophan as a function of ternary codispersions containing: a, palmitic acid; b, 11,12-dibromostearic acid; c, 9,10-dibromostearic acid; d, 2-bromopalmitic acid; and e, 6,7-dibromostearic acid. (B) Quenching of fluorescence intensity of octyltryptophan (closed circles) and butyltryptophan (open circles) as a function of the distance of bromosubstituent in fatty acid as a component of the ternary codispersions. Conditions as in Fig. 1.

TABLE II

FLUORESCENCE LIFETIMES AND QUENCHING CONSTANTS FOR TRYPTOPHAN RESIDUE OF PHOSPHOLIPASE A_2 AND ITS COMPLEXES (25°C, pH 8.0)

System	Quencher [a]	τp (ns)	K_d (M^{-1})	K_s (M^{-1})	$k_q(\times 10^9)$ ($M^{-1} \cdot s^{-1}$)
N-Acetyltryptophanamide	S	2.70	12.1	–	4.5
Octyltryptophan	S	2.3	13	–	5.6
Octyltryptophan + DMPC	S	3.7	1.4	–	0.5
Phospholipase A_2	S	2.55	5.8	1.2	2.27
PLA + Tern (1 : 60) = 60	S	3.49	7.5	0.5	1.98
Octyltryptophan	N	1.60	4.3	–	8.9
Prophospholipase A_2	N	3.40	< 0.5	–	–
Phospholipase A_2	N	2.55	< 0.5	–	–
Octyltryptophan + Tern	N	4.02	3.1	–	0.77
PLA + Tern (1 : 60 mole ratio)	N	3.49	< 0.5	–	–

[a] S is succinimide, N is *N*-methylnicotinamide.

ments. The single tryptophan residue of phospholipase A_2 is readily accessible to the succinimide as suggested by the value of bimolecular quenching constant which is about 50% of that for *N*-acetyltryptophanamide in solution. There is little change in the accessibility of the tryptophan residue when the enzyme was complexed with ternary codispersions. This degree of accessibility of tryptophan to succinimide leads to a very surprising conclusion that the tryptophan residue is present on the surface of the enzyme in solution and when the enzyme is bound to membranes. The tryptophan in free or the bilayer-localized phospholipase A_2 is not accessible to 1-methylnicotinamide which suggests the presence of positively charged groups in its microenvironment.

Further insight into the mechanism of quenching is provided by the measurement of fluorescence life-times. There is a decrease in the average fluorescence life-time of tryptophan octyl ester in the presence of 1-methylnicotinamide and succinimide. Similar behavior is also shown by phospholipase A_2 in the presence of succinimide. A decrease in the life-time in proportion to the decrease in the fluorescence intensity is expected for a collisional quenching. On the other hand, as summarized in Table I the life-times of tryptophan fluorescence from phospholipase A_2 bound to the various ternary codispersions containing a brominated component, are essentially the same as those for the ternary codispersions without any brominated component. This suggests that the quenching by brominated lipid components is essentially due to a complex formation between these lipid components and phospholipase A_2 in the ternary codispersions [11], and not due to a collisional encounter.

Discussion

The observations summarized in the preceding section show that the tryptophan fluorescence is enhanced about 100% on binding of phospholipase A_2 to the ternary codispersions. This change in fluorescence intensity is accompanied by a relatively small change in the fluorescence life-time. As implicated for other proteins the lower quantum yield of tryptophan fluorescence in phospholipase A_2 could be due to static quenching by charged groups and peptide bonds in the vicinity of the indole ring [12,13]. In the lipid environment these interactions change. The new environment is apparently more hydrophobic because it gives higher quantum yield and a blue shift.

Quenching of the tryptophan fluorescence by the two water soluble quenchers suggests that the accessibility of tryptophan from the aqueous environment does not change significantly on incorporation of phospholipase A_2 to bilayers. However, it is intriguing that the tryptophan in phospholipase A_2 is quenched by succinimide but not by 1-methylnicotinamide. Both of these quenchers have approximately the same size. This suggests the presence of positive charges in the vicinity of Trp-3 [2,14]. Exact juxtaposition of Ala-1 is crucial for the proper functioning of the interfacial recognition site, and Ala-1 is buried in the interior of the enzyme where it interacts with Gln-4 which is an invariant residue [7,15]. However, Ala-1 does not have a charge in prophospholipase A_2, whose fluorescence is also not quenched by *N*-methylnicotinamide, therefore the charge effect must arise from some other residue. Arg-6 and Lys-10 will be in the vicinity in an α-helix.

Based on the quenching experiments with brominated lipids, we suggest that the hydrophobic environment for tryptophan is modified by the acyl chains of phospholipids that are specifically bound to the protein. As shown in Fig. 2, 1-hexadecyl-2-(9,10-dibromo-octadecyl)phosphatidylcholine, as well as 1-(9,10-dibromooctadecyl)-2-hexadecylphosphatidylcholine (Table I), are more effective quenchers than 9,10-dibromooctadecanoic acid, which is significantly more effective than 1-(9,10-dibromo-octadecanoyl)lysophosphatidylcholine. One of the simplest model, that could account for this and other data, is that the three lipids in the ternary codispersions bind to pig pancreatic phospholipase A_2 in such a way that the 9,10-position of the acyl chains of phosphatidylcholine is in close contact with the tryptophan residue.

The free fatty acid chain can also be in a close contact with the tryptophan, although somewhat less effectively than the acyl chains of diacylphosphatidylcholine. In contrast, the 9,10-position of the acyl chain in 1-acyllysophosphatidylcholine in the ternary codispersions is not in

such a direct contact. This intrinsic asymmetry of the environments of the three types of the alkyl chains in the ternary codispersions sampled by the tryptophan residue is a strong argument in favor of a specific binding interaction between phospholipase A_2 and the lipid components. This specificity could arise from either direct binding of the substrate to the active site such that when glycerophosphorylcholine moiety is near the catalytic active site, the C_9–C_{11} region of the acyl chain is near the tryptophan in the interfacial recognition region. It is also possible that the tryptophan is in the diacylphosphatidylcholine-rich region of the phase separated ternary codispersions. The first possibility appears more likely and it implies that the double bonds in C_9–C_{13} region could play a role in the recognition of the substrate by phospholipase A_2.

Acknowledgments

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