Catalytic Ca^{2+}-Binding Site of Pancreatic Phospholipase A_2: Laser-Induced Eu^{3+} Luminescence Study†

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Received June 7, 1984

ABSTRACT: $^7F_0 \rightarrow {}^5D_0$ excitation spectroscopy of Eu^{3+} has been used to study the catalytic Ca^{2+}-binding site of pancreatic phospholipases A_2. Eu^{3+} binds competitively with Ca^{2+} to the enzyme with retention of about 5% of the activity found with Ca^{2+}. The dissociation constants for the Eu^{3+}–enzyme complexes of bovine phospholipase A_2 and porcine isophospholipase A_2 are 0.22 mM and 0.16 mM, respectively. Results obtained with the porcine phospholipase A_2 at neutral pH indicate aggregation of this enzyme at protein concentrations above 0.18 mM. The Eu^{3+} bound at the catalytic site of pancreatic phospholipase A_2 is coordinated to four or five water molecules, which, in conjuction with binding constant data, suggests the involvement of two or three protein ligands. Addition of a monomeric substrate analogue to the enzyme–Eu^{3+} complex results in the loss of an additional water molecule from the first coordination sphere of the bound Eu^{3+}. This result suggests an interaction between the negative charge of the polar head group of the substrate analogue and the Eu^{3+}. Binding of the enzyme–Eu^{3+} complex to micelles results in a nearly complete dehydration of the Eu^{3+} bound to the catalytic center. In the phospholipase A_2–Eu^{3+}–micelle complex, only one H_2O molecule is coordinated to Eu^{3+}. This dehydration at the active site of phospholipase A_2 in the protein–lipid complex can be an important reason for the enhanced activity of this enzyme at lipid–water interfaces.

Phospholipases A_2 (EC 3.1.1.4) (PLA's)[1] are lipolytic enzymes, which can be found inside and outside the cell. Detailed reviews on the occurrence and properties of the extracellular PLA's have recently appeared (Slotboom et al., 1982; Volwerk & de Haas, 1982). These PLA's are small (M_r 14000), water soluble, very stable proteins that require Ca^{2+} ions for the stereospecific hydrolytic activity on 3-*sn*-phosphoglycerides. In pancreatic tissue the enzyme occurs as a zymogen that is converted into the active enzyme by limited tryptic proteolysis (de Haas et al., 1968). The active enzyme possesses, like its precursor, a low catalytic activity toward monomeric substrates. When substrate is present in certain organized lipid–water interfaces, e.g., micelles, there is a large increase in the enzymatic activity of PLA but not of its zymogen (Pieterson et al., 1974b). Only the active PLA contains a region, called the lipid-binding domain, which preferentially interacts with organized neutral lipid–water interfaces. Modification studies have revealed that the α-NH_3^+ function of Ala-1 is essential in the case of the pancreatic PLA's for a functional lipid-binding domain, which is constituted mainly by hydrophobic (Trp-3, Tyr-69, Leu-19, Leu-2) and positively charged (Arg-6, Lys-10) amino acid residues (Slotboom & de Haas, 1975; Meijer et al., 1979; van Scharrenburg et al., 1981). The X-ray structure of bovine PLA (1.7 Å) reveals that the lipid-binding domain forms a hydrophobic edge surrounding the cavity of the active site (His-48, Asp-49, Asp-99) (Dijkstra et al., 1981a,b). The catalytic Ca^{2+} ion is located in the active site (Figure 1). The dissociation constant for the porcine PLA–Ca^{2+} complex ranges from 100 mM at pH 4.0 and 2.5 mM at pH 6.0 to 0.2 mM at pH 10.0 (Slotboom et al., 1982). $K_{Ca^{2+}}$ values reported for the bovine enzyme (Fleer et al., 1981) are about 5 times higher. At neutral pH, the affinity of pancreatic PLA's for the catalytic Ca^{2+} is considerably enhanced by the presence of micellar substrate analogues, e.g., *n*-alkylphosphocholines. The catalytic Ca^{2+} ion is believed to be involved in the binding of monomeric lipid molecules to the active center (Verheij et al., 1980).

Owing to the fact that tripositive lanthanide ions, Ln^{3+}, and Ca^{2+} have similar chemical properties, Ln^{3+} ions are generally able to replace Ca^{2+} ions bound to biological macromolecules. Their utility as probe species lies in the variety of spectroscopic and magnetic properties exhibited by the 14 elements of the $4f^n$ series (Horrocks, 1982).

Of particular relevance to the present research is the laser-excited Eu^{3+} luminescence technique. This technique is based on the fact that direct excitation of f-electron levels of Eu^{3+} results in an environmentally sensitive decay of the luminescence emission. The presence of OH oscillators in the first coordination sphere of the metal ion provides an efficient pathway for radiationless deexcitation, via energy transfer to OH vibrational overtones. This pathway is virtually eliminated by replacement of H_2O molecules by D_2O or other O ligands. This special property of the Eu^{3+}–H_2O complex makes it possible to determine the number of water molecules in the first coordination shell of the metal ion. The applicability of

† This work was supported in part with financial aid from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of The Netherlands Foundation for Chemical Research (S.O.N.) and by Grant GM 23599 (to W.DeW.H.) from the National Institutes of Health of the U.S. Public Health Service.

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[1] Abbreviations: PLA, phospholipase A_2; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; C_{10}PN, *n*-decylphosphocholine; C_{18}PN, *n*-octadecylphosphocholine; $C_{18:1}$PN, *n*-*cis*-9-octadecenylphosphocholine; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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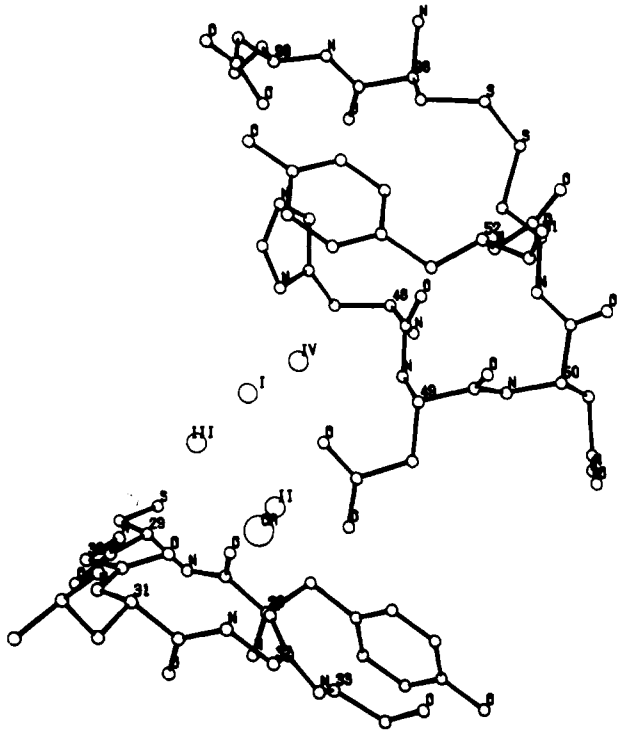

FIGURE 1: View of active site of bovine pancreatic phospholipase A_2 (Asp-99, His-48, and Asp-49), including the calcium ion and several water molecules. The Ca^{2+} is bound to the side chain of Asp-49, to the carbonyl oxygen atoms of Tyr-28, Gly-30, and Gly-32, and to two water molecules labeled II and III.

this technique to biochemical systems was clearly shown by Horrocks & Sudnick (1979a,b, 1981). In this paper this technique has been used to obtain detailed information regarding the catalytic Ca^{2+}-binding site of pancreatic PLA's, in the presence and absence of monomeric and micellar substrate analogues.

Materials and Methods

Materials. The pancreatic pro- and isoprophospholipases A_2 from pig and ox were purified from pancreatic tissue and converted into phospholipases A_2 (PLA's) by limited proteolysis as described by Nieuwenhuizen et al. (1974), van Wezel & de Haas (1975), and Fleer et al. (1978), respectively. 1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine was prepared as described by Cubero Robles & van den Berg (1969). *n*-Decylphosphocholine, *n*-octadecylphosphocholine, and *cis*-9-octadecenylphosphocholine were synthesized as described by van Dam-Mieras et al. (1975). $EuCl_3 \cdot 6H_2O$ was obtained from Alfa-Ventron Chemicals. Deuterium oxide (99.9%) was purchased from Sigma Chemical Co., and all other chemicals were reagent grade or the purest commercially available.

Methods. Protein concentrations were calculated from the absorbance at 280 nm with E_{1cm} (1%) values of 12.3 for bovine PLA and of 13.0 for porcine PLA and iso-PLA. Kinetic measurements with micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine were performed essentially as described before (de Haas et al., 1971). The concentrations of Eu^{3+} solutions were determined by EDTA titration, employing arsenazo as indicator (Woyski & Harris, 1963).

Eu^{3+} Excitation Spectra of the $^7F_0 \rightarrow {}^5D_0$ Transition. The solutions of the different PLA's (40 μM) in 10 mM piperazine buffer, pH 6.0, containing 0.1 M NaCl and 40 μM Eu^{3+}, were irradiated with a pulsed nitrogen laser pumped dye laser system, described elsewhere (Horrocks & Sudnick, 1981), and continuously scanned from 578.00 to 580.00 nm (Rhodamine 6G dye). The emission intensity at 615 nm was recorded as a function of excitation wavelength with the aid of a cooled photon-counting system and multichannel analyzer.

Eu^{3+} Titration of Pancreatic Phospholipases A_2 Monitored by $^7F_0 \rightarrow {}^5D_0$ Excitation at 579.00 nm. The Eu^{3+} binding to the catalytic Ca^{2+}-binding site of PLA was measured by titrating increasing aliquots of PLA solutions (25 mM Hepes, 0.1 M NaCl, 40 μM Eu^{3+}) at pH 7 and 25 °C to a solution of 40 μM Eu^{3+} in the same buffer. The increase in emitted photons ($^5D_0 \rightarrow {}^7F_2$, 615 nm), a result of the laser-induced excitation of Eu^{3+} at 579.00 nm, was plotted as a function of the protein concentration. The dissociation constants (K_d) of the PLA–Eu^{3+} complexes were calculated as follows. The Eu^{3+} luminescence data obtained with bovine PLA and porcine iso-PLA were used as input for a nonlinear regression analysis based on the method of Fletcher & Powell (1963), involving a combination of the steepest descent and Gauss–Newton regression methods (Hille et al., 1981). Data input consists of values for total Eu^{3+} concentration ($[Eu^{3+}_T]$), total PLA concentration ($[PLA_T]$), and the corresponding intensity (Q_{obsd}). The data are fitted using eq 1, where K_d represents

$$K_d = \frac{([Eu^{3+}_T] - X)([PLA_T] - X)}{X} = \frac{[Eu^{3+}][PLA]}{[PLA\text{–}Eu^{3+}]} \qquad (1)$$

the dissociation constant of the PLA–Eu^{3+} complex and X is the concentration of the complex. The stoichiometry of the PLA–Eu^{3+} complexes was taken 1:1, as determined already by Pieterson et al. (1974a) for Ca^{2+} binding and used by Hershberg et al. (1976) for Gd^{3+} binding. Equation 1, in fact a quadratic function in X, is solved for X (yielding only one possible root) by using an initial guessed value for K_d. A calculated signal, Q_{calcd}, is then obtained from the relation: $Q_{calcd} = CX + ([Eu^{3+}_T] - X)C_{Eu^{3+}(aq)}$, where C is also an initial guessed value that represents the luminescence intensity at 615 nm per micromole of bound Eu^{3+} (arbitrary units). A correction is made for the contribution of free Eu^{3+}(aq). $C_{Eu^{3+}(aq)}$ represents the luminescence intensity at 615 nm per micromole of free Eu^{3+}(aq) and is based on the initial point [no added protein, 40 μM Eu^{3+}(aq)]. The program searches for the minimum of the summed squared difference $[\sum^n(Q_{obsd} - Q_{calcd})^2]$ over all n data points. When a minimum is found, the parameters K_d and C are given as output, together with the covariance and standard deviations.

Competition Binding between Eu^{3+} and Ca^{2+}. Competition between Eu^{3+} and Ca^{2+} for the catalytic Ca^{2+}-binding site of the bovine and porcine PLA's and porcine iso-PLA was measured by adding aliquots of a concentrated $CaCl_2$ solution (1 M) to a solution of $EuCl_3$ (40 μM) and enzyme (500 μM) in Hepes buffer of pH 7.0 (0.1 M NaCl, 25 °C). The laser-induced Eu^{3+} luminescence intensity was plotted vs. the Ca^{2+} concentration. The dissociation constants of the bovine PLA–Ca^{2+} and porcine iso-PLA–Ca^{2+} complexes were computed from the competitive binding data with the known K_D values of the enzyme–Eu^{3+} complexes.

Determination of Number of Water Molecules on Eu^{3+} Bound to the Catalytic Ca^{2+}-Binding Site of Pancreatic Phospholipases A_2 in the Absence and Presence of Substrate Analogues. All experiments were performed with 0.1–1.0 mM PLA solutions in 25 mM Hepes buffer (pH 7.0) containing 40 μM Eu^{3+} and 0.1 M NaCl (25 °C). The reciprocal lifetime (τ^{-1}) of excited Eu^{3+} bound to the different PLA's was measured in the presence and absence of monomeric $C_{10}PN$ (8 mM), micellar $C_{18}PN$ (50 mM), and micellar $C_{18:1}PN$ (50 mM). The value of the reciprocal lifetime of Eu^{3+} bound to the PLA's under the same conditions in pure D_2O was also determined. Measurement of the τ^{-1} of Eu^{3+} in the difference

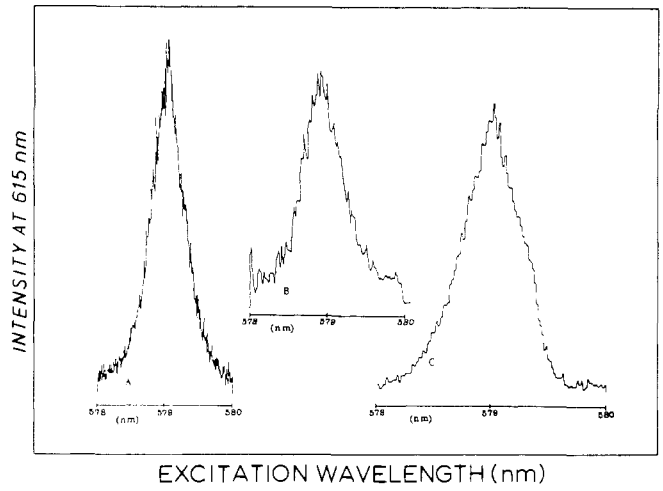

FIGURE 2: $^7F_0 \rightarrow {}^5D_0$ excitation spectra of pancreatic phospholipase A_2–Eu^{3+} complexes (20 μM Eu^{3+} and 40 μM pancreatic PLA in 10 mM piperazine buffer, pH 6.0): (A) bovine PLA–Eu^{3+}; (B) porcine iso-PLA–Eu^{3+}; (C) porcine PLA–Eu^{3+}.

samples was achieved by irradiating the samples with the pulsed dye laser at 579.00 nm while monitoring the decay of luminescence at 615 nm with the photon-counting system and multichannel analyzer. By use of the equation $q = 1.05(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O})$, the number of water molecules (Horrocks & Sudnick, 1979a,b, 1981) coordinated to Eu^{3+} in the different enzyme–Eu^{3+}–*n*-alkylphosphocholine complexes was calculated. A more detailed description of the instrumentation can be found elsewhere (Sudnick, 1979; Horrocks & Sudnick, 1981).

Results

Enzymatic Activity. The maximum velocity of the bovine, PLA-catalyzed, porcine iso-PLA-catalyzed, and porcine PLA-catalyzed hydrolysis of micellar dioctanoyllecithin in the presence of Ca^{2+} (5 mM) is about 2000 μmol/(min·mg). The corresponding maximum velocity in the presence of Eu^{3+} (5 mM) under the same conditions is about 100 μmol min^{-1} (mg of protein)$^{-1}$ for all three phospholipases A_2, while no activity could be detected in the absence of Eu^{3+}.

Eu^{3+} Titration of Phospholipase A_2 via $^7F_0 \rightarrow {}^5D_0$ Excitation Spectroscopy. The $^7F_0 \rightarrow {}^5D_0$ excitation spectra of bovine PLA and porcine iso-PLA at pH 6.0 both show one single symmetrical peak centered around 579.00 nm with a width at half-height of 0.55 nm (spectra A and B of Figure 2, respectively). This peak is attributed to Eu^{3+} binding to the single, catalytically active, binding site in these PLA's. This contention is supported by the fact that the Eu^{3+}-substituted protein retains some catalytic activity and that free Eu^{3+} aqua ion yields a peak centered at 578.8 nm that is very difficult to observe under these low concentration conditions (Horrocks & Sudnick, 1979b). The excitation spectrum of porcine phospholipase A_2 shows an additional feature on the low-energy side (Figure 2C). The width at half-height of the composite peak is 0.67 nm. Although the porcine PLA possesses a second Ca^{2+}-binding site, it is unlikely that the low-energy feature is due to binding of Eu^{3+} to this low-affinity site ($K_d \approx 50$ mM for Ca^{2+} at pH 6.0) (Donné-Op den Kelder et al., 1983). More probably, this second component is a result of Eu^{3+} bound to the catalytic Ca^{2+} site of porcine PLA present in an aggregated form (see Discussion).

Titrations of Eu^{3+} with bovine PLA, porcine iso-PLA, and porcine PLA are shown in curves A–C of Figure 3, respectively. The K_d values for the enzyme–Eu^{3+} complexes of bovine PLA and porcine iso-PLA of 0.22 mM and 0.16 mM, respectively, were obtained from an analysis of the data in Figure 3A,B. It turned out to be impossible to determine the exact K_d value for the Eu^{3+}–porcine PLA complex. At higher concentrations of protein the signal of the Eu^{3+}–enzyme complex monitored via excitation at 579.00 nm decreased, due to a shift of the maximum of the excitation spectrum to higher wavelengths. As the concentration of porcine PLA is increased, the contribution of the second low-energy component to the excitation grows at the expense of the component at 579.00 nm. The alternative titrations of PLA solutions with Eu^{3+} failed because saturation of the signal could not be obtained. At concentrations above approximately 100 μM Eu^{3+}, a monotonous increase in the emission signal with the Eu^{3+} concentration is observed, which is likely due to aspecific binding of Eu^{3+} to the PLA's.

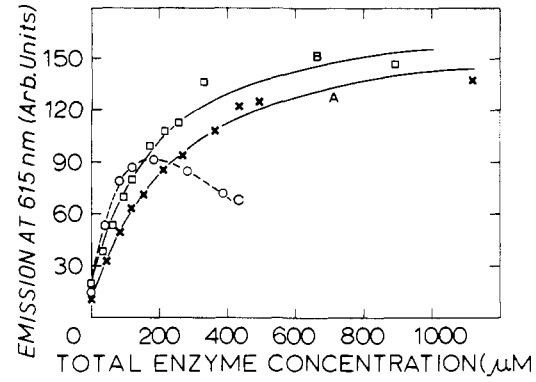

FIGURE 3: Eu^{3+} luminescence titration curves of bovine phospholipase A_2 [(A) $K_d = 0.22 \pm 0.02$ mM], porcine isophospholipase A_2 [(B) $K_d = 0.16 \pm 0.02$ mM], and porcine phospholipase A_2 (C). Excitation was at 579 nm with monitoring of the emission at 615 nm. Conditions were 40 μM Eu^{3+}, 0.1 M NaCl, and 25 mM Hepes, pH 7.0, at 25 °C.

Competitive Binding Experiments. Competition experiments between the Eu^{3+} and Ca^{2+} for the catalytic Ca^{2+}-binding site were carried out for bovine PLA and porcine iso-PLA. The decrease of the luminescence of the Eu^{3+} upon addition of concentrated Ca^{2+} solution clearly showed that the Eu^{3+} is replaced by the Ca^{2+} ion. The dissociation constant of the enzyme–Ca^{2+} complexes of bovine PLA and porcine iso-PLA calculated from these titration data were 4.7 mM and 1.4 mM, respectively. These values are in good agreement with $K_{Ca^{2+}}$ values obtained directly for bovine PLA and porcine iso-PLA (Fleer et al., 1981; van Wezel et al., 1976).

Determination of Number of Water Molecules Coordinated to Eu^{3+} Bound to Pancreatic PLA–(n-Alkylphosphocholine) Complexes. The reciprocal lifetime data and the derived numbers of water molecules in the first coordination sphere of Eu^{3+} present in the different complexes with pancreatic PLA's and *n*-alkylphosphocholines are shown in Table I. In the absence of lipid, the Eu^{3+} ion bound to the catalytic site in porcine PLA and iso-PLA is coordinated with 4.6 and 4.1 H_2O molecules, respectively. Under the same conditions, 5.2 water molecules are coordinated to the Eu^{3+} present on the metal ion binding site of bovine PLA.

Addition of the monomeric substrate analogue *n*-decylphosphocholine to the PLA–Eu^{3+} complexes leads to the exclusion of about one additional water molecule in all three cases. Binding of all three PLA's to the micellar lipid *n-cis*-9-octadecenylphosphocholine has a dramatic effect on the number of water molecules coordinated to the catalytic Eu^{3+}. Only 0.8–1.2 H_2O molecules remain coordinated, which implies a severe dehydration of the metal ion at the active site in the Eu^{3+}–PLA–micelle complex. The iso-PLA–Eu^{3+} complex in the presence of $C_{18}PN$ micelles gives nearly the same

Table I: Number of Water Molecules Coordinated to Eu^{3+} Bound to Phospholipase A_2 in the Absence and Presence of *n*-Alkylphosphocholines

	porcine PLA		porcine iso-PLA		bovine PLA	
	τ^{-1} (ms^{-1})[a]	q[b]	τ^{-1} (ms^{-1})	q	τ^{-1} (ms^{-1})	q
Eu^{3+}–PLA in D_2O	0.80		0.83		0.75	
Eu^{3+}–PLA	5.20	4.6	4.69	4.1	5.68	5.2
Eu^{3+}–PLA–monomeric $C_{10}PN$	3.84	3.2	3.52	2.8	4.86	4.3
Eu^{3+}–PLA–micellar $C_{18}PN$	3.33	2.7	1.47	0.7	2.46	1.8
Eu^{3+}–PLA–micellar $C_{18:1}PN$	1.62	0.9	1.63	0.8	1.97	1.3

[a] τ^{-1} values are the luminescence decay constants of excited Eu^{3+} in H_2O, unless indicated otherwise. Excitation wavelength was 579.00 nm. [b] q is the number of H_2O molecules coordinated to Eu^{3+} and was calculated by using the equation of Horrocks & Sudnick (1979a, 1981): $q = 1.05(\tau^{-1}_{H_2O} - \tau^{-1}_{D_2O})$. All values are ±0.5 due to the uncertainty of the correlation between the luminescence decay values and the actual number of water molecules (Horrocks & Sudnick, 1979a).

result as in the presence of the unsaturated substrate analogue (Table I). This is not the case with the bovine enzyme, which is known to possess a lower affinity for lipid–water interfaces (van Scharrenburg et al., 1981; Dutilh et al., 1975). Under the conditions used, not all the bovine PLA will be bound to the $C_{18}PN$ micelles. This is reflected in the higher number of H_2O molecules coordinated to the Eu^{3+} in the bovine PLA sample in the presence of $C_{18}PN$ as compared to $C_{18:1}PN$. It is known that the affinity of pancreatic PLA's for micelles of $C_{18:1}PN$ is higher than for micelles of $C_{18}PN$, due to the presence of unsaturation in the alkyl chain of the former. The difference between the number of H_2O molecules coordinated to Eu^{3+} in the Eu^{3+}–porcine PLA–micellar $C_{18}PN$ sample and in the Eu^{3+}–porcine PLA–micellar $C_{18:1}PN$ sample is even greater than that observed for the bovine PLA. This difference indicates that at pH 7.0 there is almost no binding to the saturated substrate analogue. Donné-Op den Kelder et al. (1981, 1983) have previously shown that porcine PLA needs a Ca^{2+} ion bound to the second, low-affinity Ca^{2+}-binding site for optimal binding to lipid–water interfaces at basic pH.

The data of Table I for porcine PLA were obtained with a 0.2 mM protein solution instead of the 1.0 mM solutions employed for the other two PLA's. At this lower concentration (0.2 mM) the number of water molecules coordinating to Eu^{3+} in the different complexes with porcine PLA is in agreement with those obtained for porcine iso-PLA.

At high concentrations of porcine PLA (1 mM) where the low-energy component in the excitation spectrum is considerable, the following reciprocal lifetime results were obtained: $\tau^{-1}_{H_2O} = 3.33$ ms^{-1} and $\tau^{-1}_{D_2O} = 0.43$ ms^{-1}. This indicates that the number of water molecules coordinated to the Eu^{3+} bound to porcine PLA, in the absence of lipid, decreases from 4.6 to 3.0 upon an increase of the protein concentration from 0.2 to 1.0 mM. This result indicates that this low-energy component arises from Eu^{3+} coordinated to at least one fewer water molecule. In any case, the Eu^{3+} seems to be in contact with an additional protein ligand. In the presence of micellar $C_{18:1}PN$ no difference is observed in the number of coordinated H_2O molecules between 1.0 and 0.2 mM porcine PLA samples.

Discussion

Our findings that Eu^{3+} competes with Ca^{2+} for occupation of the active catalytic site and, in the absence of Ca^{2+}, supports 5% of the activity of native pancreatic PLA's toward micellar short-chain lecithin attest to the validity of the use of Eu^{3+} as a substitutional probe at the catalytic center. Gd^{3+} has also been reported (Hersberg et al., 1976) to support catalytic activity in PLA. Although the Eu^{3+}-activated lipolysis represents a 20-fold decrease from that supported by Ca^{2+}, this activity is still 20–30 times that of the zymogen toward the same substrate. It may be that the decreased activity in the case of Eu^{3+} is related to its lower rate of ligand exchange. Water exchange rates for Ln^{3+} and Ca^{2+} ions are approximately 10^7 s^{-1} and 5×10^9 s^{-1}, respectively (Eigen, 1963). The higher positive charge of Eu^{3+} compared to Ca^{2+} is also responsible for the 10–20-fold greater affinity of the former ion for binding to PLA. The $K_d(Eu^{3+})$ values extracted from the luminescence titration data (0.22 mM and 0.16 mM for bovine PLA and porcine iso-PLA, respectively) are in reasonable accord with those obtained by Hershberg et al. (1976) from proton resonance relaxation data at pH 5.8 [$K_d(Gd^{3+}) = 0.45$ mM; $K_d(Tb^{3+}) = 0.08$ mM].

Probably the most significant results of the present study concern the changes in the number of water molecules coordinated at the active site of PLA's under various conditions. Our data suggest that free in solution at lower concentrations bovine and porcine PLA have about five water molecules coordinated to the Eu^{3+} ion, while porcine iso-PLA has four such water molecules. At first sight this result appears to be at odds with the structural details for the Ca^{2+}–PLA complexes obtained by X-ray diffraction methods (Figure 1) (Dijkstra et al., 1981b, 1983). Two factors may account for the difference in the number of water molecules observed to be coordinated to Eu^{3+} and Ca^{2+}. The first is that Eu^{3+} may in fact accommodate one more water molecule than Ca^{2+}. This was observed (Matthews & Weaver, 1974) to occur upon substitution of a Eu^{3+} ion for Ca^{2+} at calcium site 4 of thermolysin. The second, and likely the primary reason for the apparent discrepancy, is that the X-ray structure represents the molecule in a condensed phase. Our results reveal a marked tendency toward metal ion dehydration upon protein aggregation at higher concentrations or interaction with lipid phases. Apparently, the catalytic metal ion binding sites of pancreatic PLA's appear to be flexible and sensitive to the state of aggregation of the enzyme. The predominant Eu^{3+} aqua ion in solution involves nine coordinated water molecules. Our results suggest that four or five of these are displaced upon the binding of Eu^{3+} to PLA's. Surveys of the protein X-ray structural literature (Horrocks, 1982; Kretsinger & Nelson, 1976) reveal that the total coordination numbers of Ca^{2+} and Ln^{3+} ions bound to proteins lie in the range of 6–8. Thus, our finding that four or five water molecules remain coordinated to the PLA-bound Eu^{3+} ion implies that one to four protein-provided ligand atoms are involved in sequestering the metal ion under these conditions. The magnitudes of the measured dissociation constants for Eu^{3+} ($K_d \sim 0.2$ mM) are most consistent with their being two or three protein-supplied ligating groups when comparison is made with stability constant data in the literature (Sillén & Martell, 1971a,b), for ligands of various known dentisities. It is likely that only in condensed or associated phases do all four of the ligand moieties noted in the X-ray structure become involved in the metal ion binding.

Addition of the monomeric substrate anlogue, which binds in a 1:1 molar ratio to pancreatic PLA–Eu^{3+} complexes (Volwerk et al., 1979), results in a decrease of approximately one coordinated water molecule. This result lends strong

support to the proposed catalytic mechanism of PLA (Verheij et al., 1980), which assumes the negative phosphate group to be directly coordinated to the catalytic Ca^{2+} ion with the remaining part of the head group exposed to the solvent. In the case of a real substrate, the Ca^{2+} is also suggested to interact with the carbonyl of the 2-ester bond of the phospholipid molecule. This structure is postulated to stabilize the tetrahedral intermediate formed by a nucleophilic attack of a water molecule on the 2-ester bond.

The extensive dehydration of the Eu^{3+} bound to the active site of pancreatic PLA in the presence of micelles is the most striking result of the present study. When the enzyme is embedded in a lipid–water interface only one of the ligands of the Eu^{3+} at the catalytic center of PLA is a water molecule. Wells and colleagues (Misiorowski & Wells, 1974; Poon & Wells, 1974; Wells, 1974) noted that the activity of PLA is very sensitive to changes in lipid hydration. Brockerhoff (1968) suggested as early as 1968 that the high activity of lipolytic enzymes at lipid–water interfaces compared to monomeric lipids is due to desolvation of the substrate at the interface, which allows a more successful attack of a weak nucleophile such as a water molecule. The presence of only one water molecule coordinated to the catalytic Eu^{3+} points to a highly dehydrated active site of PLA when bound to a lipid–water interface. However, whether this dehydration alone is responsible for the high enzymatic activity toward neutral lipid-water interfaces or, alternatively, whether the enhanced activity can be explained by a conformational change (induced by the lipid–water interface) is still an open question.

The anomalous results obtained with concentrated porcine PLA solutions compared with porcine iso-PLA and bovine PLA under the same conditions at pH 7.0 can be explained by partial aggregation or porcine PLA in the absence of micellar substrate analogues. Also, NMR studies showed that specifically the porcine PLA aggregates at high protein concentrations above pH 5.0 (Aguiar et al., 1979; Jansen, 1979).

The shift of the excitation maximum of the Eu^{3+} luminescence to higher wavelength and the decrease of the number of water molecules coordinated to Eu^{3+} bound at high porcine PLA concentrations suggest another type of binding site for the Eu^{3+}. An attractive possibility is that the Eu^{3+} is sandwiched in between the catalytic sites of two protomers. Such a structure very much resembles that of the *Crotalus atrox* PLA dimer, in which the active sites are facing each other (Keith et al., 1981). Intriguing also is the fact that porcine PLA shows this aggregation behavior at high concentrations while the isoenzyme does not, although these enzymes differ only in four amino acid residues. The presence of a histidine residue at position 17, located in the lipid binding domain of the porcine PLA, instead of Asp in the porcine iso-PLA might be responsible for this effect. Deprotonation of His-17 will create an additional uncharged aromatic residue in the lipid-binding domain that might favor protein aggregation. In any case, at the protein concentrations normally used for kinetic and binding studies, this aggregation of porcine pancreatic PLA will not play a significant role.

Registry No. PLA, 9001-84-7; $C_{10}PN$, 70504-28-8; $C_{18}PN$, 65956-63-0; $C_{18:1}PN$, 76622-80-5; Ca, 7440-70-2; Eu, 7440-53-1.

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Influence of Cofactor Pyridoxal 5′-Phosphate on Reversible High-Pressure Denaturation of Isolated β_2 Dimer of Tryptophan Synthase Bienzyme Complex from *Escherichia coli*†

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Received July 2, 1984

ABSTRACT: High hydrostatic pressure has been shown to cause reversible dissociation of the isolated apo β_2 dimer of tryptophan synthase from *Escherichia coli* into enzymatically inactive monomers [Seifert, T., Bartholmes, P., & Jaenicke, R. (1982) *Biophys. Chem. 15*, 1–8]. Addition of the coenzyme pyridoxal 5′-phosphate affects the structural stability, as well as the kinetics of dissociation and deactivation. The apo β_2 dimer is deactivated faster than the holoenzyme by a factor of 10. The midpoints of the corresponding equilibrium transition curves are observed at 690 and 870 bar, respectively. As shown by hybridization of native and chemically modified β chains, the loss of enzymatic activity is accompanied by subunit dissociation. An additional deactivating effect is produced by the pressure-induced release of the cofactor from the holoenzyme. Renaturation after decompression has been monitored by circular dichroism and intrinsic fluorescence emission. Alterations of the dichroic absorption at 222 nm reflect the recovery of the native secondary structure, while tryptophan fluorescence represents a specific probe for the native tertiary structure in the immediate neighborhood of the active center of the enzyme. By application of both methods to monitor the reconstitution of the apo β_2 dimer, two first-order processes may be separated along the time scale. The faster phase ($k_1 = 1.2 \times 10^{-2}$ s^{-1}) yields a "structured monomer" with 85% native secondary structure and the tryptophan side chain buried in its native hydrophobic environment. As indicated by sodium borohydride reduction, this intermediate is able to interact with the coenzyme pyridoxal 5′-phosphate in the correct way; however, it does not show enzymatic activity. During the second phase ($k_1' = 8.3 \times 10^{-4}$ s^{-1}), a slow reshuffling process provides properly folded contact regions for rapid dimerization of the inactive monomers resulting in the catalytically active native holoenzyme.

The biological function of proteins (enzymes) depends on the unique spatial arrangement of their polypeptide backbone. This gains its stability from noncovalent short-range and long-range forces including hydrogen bonds, ion pairs, and hydrophobic interactions. Experiments making use of reversible denaturation and reconstitution (Anson, 1945; Anfinsen et al., 1961) clearly show that the one-dimensional amino acid sequence contains the "code" for the three-dimensional folding of the polypeptide chain in its aqueous or nonaqueous environment. The underlying folding mechanism involves an ordered sequence of steps proceeding from short-lived microdomains to increasingly higher substructures, which finally undergo tertiary structure formation due to long-range interactions between preformed elements of secondary structure. The time scale for the various intermediate states on the pathway of folding ranges from microseconds (helix formation) to minutes ("reshuffling") (Ko et al., 1977; Baldwin, 1980; Weaver, 1982; Burns & Schachman, 1982; Jaenicke, 1984).

The reconstitution of oligomeric proteins includes specific homologous or heterologous interactions of protein subunits. Since it requires the correct formation of subunit contact regions, association reactions must be late events on the

† Dedicated to Professor Max A. Lauffer on the occasion of his 70th birthday. This work was supported by grants of the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft to P.B. and R.J.
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0006-2960/85/0424-0339$01.50/0