Galardy, R. E., & Kortylewicz, Z. P. (1985) *Biochemistry* (in press).
Gallop, P. M., Seifter, S., & Meilman, E. (1957) *J. Biol. Chem. 227*, 891–906.
Gordon, E. M., Natarajan, S., Pluscec, J., Weller, H. N., Godfrey, J. D., Rom, M. B., Sabo, E. F., Engebrecht, J., & Cushman, D. W. (1984) *Biochem. Biophys. Res. Commun. 124*, 148–155.
Hangauer, D. G., Monzingo, A. F., & Matthews, B. W. (1984) *Biochemistry 23*, 5730–5741.
Kester, W. R., & Matthews, B. W. (1977) *J. Biol. Chem. 252*, 7704–7710.
Lewis, C. A., & Wolfenden, R. (1977a) *Biochemistry 16*, 4890–4895.
Lewis, C. A., & Wolfenden, R. (1977b) *Biochemistry 16*, 4886–4890.
Lwebuga-Mukasa, J. S., Harper, E., & Taylor, P. (1976) *Biochemistry 15*, 4736–4741.
MacKenzie, N. E., Malthouse, J. P. G., & Scott, A. I. (1984) *Science (Washington, D.C.) 225*, 883–889.
McCroskery, P. A., Richards, J. F., & Harris, E. D. (1975) *Biochem. J. 152*, 131–142.
Meyer, R. F., Nicolaides, E. D., Tinney, F. J., Lunney, E. A., Homes, A., Hoefler, M. L., Smith, R. D., Essenburg, A. D., Kaplan, H. R., & Almquist, R. G. (1981) *J. Med. Chem. 24*, 964–969.
Natarajan, S., Gordon, E. M., Sabo, E. F., Godfrey, J. D., Weller, H. N., Pluscec, J., Rom, M. B., & Cushman, D. W. (1984) *Biochem. Biophys. Res. Commun. 124*, 141–147.
Quiocho, F. A., & Lipscomb, W. N. (1971) *Adv. Protein Chem. 25*, 1–78.
Rees, D. C., Honzatko, R. B., & Lipscomb, W. N. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3288–3291.
Rich, D. H., Bernatowicz, M. S., & Schmidt, P. G. (1982) *J. Am. Chem. Soc. 104*, 3535–3536.
Schechter, I., & Berger, A. (1968) *Biochem. Biophys. Res. Commun. 32*, 898–902.
Seifter, S., & Harper, F. (1971) *Enzymes (3rd Ed.) 3*, 649–697.
Shapiro, R., & Riordan, J. F. (1984) *Biochemistry 23*, 5234–5240.
Steinbrink, D. R., Bond, M. D., & Van Wart, H. E. (1985) *J. Biol. Chem. 260*, 2771–2776.
Sugasawara, R., & Harper, E. (1984) *Biochemistry 23*, 5175–5181.
Van Wart, H. E., & Steinbrink, D. R. (1981) *Anal. Biochem. 113*, 356–365.
Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.
Wolfenden, R. (1969) *Nature (London) 223*, 704–705.
Yagisawa, S., Morita, F., Nagai, Y., Noda, H., & Ogura, Y. (1965) *J. Biochem. (Tokyo) 58*, 407–416.

Aggregation Studies on Fluorescein-Coupled Cobra Venom Phospholipase A_2[†]

Theodore L. Hazlett and Edward A. Dennis*
Department of Chemistry, University of California at San Diego, La Jolla, California 92093
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ABSTRACT: Phospholipase A_2 from *Naja naja naja* venom (Indian cobra) undergoes a concentration-dependent aggregation, and at an assay concentration of 1 μg mL^{-1}, it exists as a monomer. However, there is some evidence that the enzyme is actually active as a dimer or higher order aggregate. Previous attempts to determine the aggregation state of the enzyme under actual assay conditions were thwarted by experimental difficulties due in part to the low enzyme concentrations required. This aggregation has now been studied by using fluorescence polarization. The extrinsic probe fluorescein isothiocyanate was coupled to the enzyme to serve as the fluorescence marker. Steady-state polarization measurements were made to determine changes in the aggregation state of the fluorescently tagged enzyme. The phospholipases A_2 from *Crotalus adamanteus* (rattlesnake) and porcine pancreas, whose states of aggregation are known, were also labeled with fluorescein isothiocyanate and used as controls. It was found that the divalent metal ions Ca^{2+}, a phospholipase cofactor, and Ba^{2+}, an inhibitor, caused an increase in the cobra venom enzyme polarization, while Mn^{2+}, Mg^{2+}, and Co^{2+} did not. The water-soluble substrate diheptanoylphosphatidylcholine and the lipid analogue dodecylphosphocholine, when present below their respective critical micelle concentrations, also increased the polarization of the phospholipase–fluorescein conjugate. Thus, both cofactor and substrate caused an increase in the polarization, which implies an increase in the aggregation state. It is concluded that under assay conditions the phospholipase A_2 exists in an aggregated form.

Phospholipase A_2 catalyzes the hydrolysis of the *sn*-2 fatty acid of phospholipids with Ca^{2+} required as a cofactor (Dennis, 1983). The enzyme from cobra venom (*Naja naja naja*) appears to have two phospholipid binding sites, an activator site and a catalytic site (Roberts et al., 1979; Adamich et al., 1979; Plückthun & Dennis, 1982). A phosphocholine-containing lipid must be bound to the activator site in order to achieve maximal enzymatic activity. Earlier experiments showed that the enzyme from cobra venom undergoes a concentration-dependent aggregation in the absence of substrate (Deems & Dennis, 1975). However, at an assay concentration of 1 μg mL^{-1}, the enzyme is a monomer. The presence of Ca^{2+} or substrate could cause the enzyme to aggregate. Studies involving gel filtration, cross-linking, and ultracentrifugation suggested that upon contact with substrate the enzyme aggregates (Roberts et al., 1977a,b; Lewis et al., 1977; Dennis

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* Address correspondence to this author. He is a Guggenheim Fellow, 1983–1984.

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et al., 1981). Although these experiments suggested that the active enzyme was in a dimeric or aggregated form, a direct demonstration of aggregates in the presence of substrate at assay concentrations of enzyme was not forthcoming.

Since fluorescence polarization is capable of extracting information about macromolecular size and shape (Weber, 1952a,b; Lakowicz, 1983), it is a convenient technique for studying aggregation. The extrinsic probe fluorescein isothiocyanate (FITC)[1] has been used to label proteins for fluorescence for polarization experiments (Chryssomallis et al., 1981) and has sufficient sensitivity to study assay concentrations of the enzyme. We have now utilized this probe to label the cobra venom phospholipase A_2 in order to directly study its aggregation state as a function of enzyme, substrate, and divalent metal ion concentrations. These results were compared with similarly labeled porcine pancreas and *Crotalus adamanteus* venom phospholipases whose states of aggregation have been well characterized. The pancreatic enzyme is a monomer of 14 000 daltons in the presence or absence of monomeric phospholipid substrate (Soares de Araujo et al., 1979); the *Crotalus adamanteus* phospholipase A_2 is a dimer of 30 000 daltons and appears to remain a dimer under most conditions (Wells, 1971; Smith & Wells, 1981). The pancreatic enzyme at 14 000 daltons serves as a control for a monomeric cobra venom phospholipase while the *C. adamanteus* enzyme serves as the control for a dimer.

Experimental Procedures

Materials. Phospholipase A_2 was purified from *N. naja naja* venom (Miami Serpentarium) as summarized elsewhere (Deems & Dennis, 1981; Hazlett & Dennis, 1985). Porcine pancreatic phospholipase A_2 was obtained from Boehringer Mannheim (lot 1382524), and *C. adamanteus* phospholipase A_2 was obtained from Worthington (lot ZOB484Y). Fluorescein isothiocyanate was obtained from Molecular Probes as a 10% mixture with Celite. Sephadex G-25 and PD-10 columns were obtained from Pharmacia Fine Chemicals. Diheptanoylphosphatidylcholine (diheptanoyl-PC) was obtained from Avanti Biochemicals. Dodecylphosphocholine (DPC) was a gift from Dr. Stuart Hendrickson (St. Olafs College, Northfield, MN). Egg phosphatidylcholine was purified by the procedure of Singleton et al. (1965).

Phospholipase A_2 Assay. Phospholipase A_2 was assayed with the pH-stat technique (Dennis, 1973). The assay mix contained 10 mM $CaCl_2$, 20 mM Triton X-100, and 5 mM egg PC for the two venom enzymes while an egg yolk assay mix, as described by Nieuwenhuizen et al. (1974), was used to assay the pancreatic phospholipase A_2.

Fluorescein–Enzyme Conjugates. Coupling of fluorescein isothiocyanate (FITC) to the three enzymes was performed at room temperature in 50 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$. Routinely, the enzymes were prepared as 1 mg mL^{-1} solutions to which a 10% FITC–Celite mixture was added to a final concentration of 10 mg mL^{-1}. The samples were then shaken for 5–15 min, and the reactions were stopped by the addition of either 100 mM β-mercaptoethanol or 10 mM cysteine. Samples were then spun to remove the Celite, and the supernatant was loaded on a series of two Sephadex G-25 columns. The first column, to remove free FITC from protein, was equilibrated and run in 6 M urea and 20 mM Tris-HCl, pH 8.0. The second column, to remove urea, was equilibrated and run in deionized water. The fractions containing FITC conjugate were then filtered through a 0.2-μm pore size Millipore filter. The *C. adamanteus* enzyme dissociates in the presence of 6 M urea; therefore, the free FITC was separated by using less stringent conditions. A single Sephadex G-25 column was equilibrated and run in 100 mM NaCl, 1 mM EDTA, and 15 mM Tris-HCl, pH 8.0. After either procedure, the fractions containing FITC–phospholipase conjugate were dialyzed against the column buffer. The concentration of bound FITC was determined by the absorbance using a molar extinction coefficient of 42 500 M^{-1} cm^{-1} at 495 nm (Tengerdy & Chang, 1966). No correction was made for decreases in absorbance due to FITC fluorescence.

Protein Determination. The reported extinction coefficients for the pancreatic, $E^{1\%}_{280} = 12.5$ (Hille et al., 1981), the *N. naja naja*, $E^{1\%}_{278} = 22.0$ (Darke et al., 1980), and the *C. adamanteus*, $E^{1\%}_{280} = 22.7$ (Wells & Hanahan, 1969), phospholipases A_2 were used to determined the protein concentrations for the unlabeled enzymes. Protein concentrations for the enzyme–fluorescein conjugates were measured by the procedure of Lowry et al. (1951). Neither free FITC nor bound FITC interfered with this procedure (G. Fortes, personal communication). Bovine serum albumin was used as the routine protein standard, and correction factors for each enzyme with respect to this standard were determined and used to convert the Lowry values to the true protein concentrations (Darke et al., 1980).

Steady-State Polarization Measurements. Fluorescence polarization was measured by using a water-jacketed Perkin-Elmer MPF-44A fluorescence spectrometer with a Perkin-Elmer polarization accessory. Corrections for instrumental polarization were carried out with horizontally polarized excitation as outlined in the polarization accessory manual. The excitation wavelength was set at 490 nm with a 5-nm band-pass, and the emission was set at 520 nm with a 10-nm band-pass. The amount of light scattering and stray light during the polarization experiments was determined in each experiment by including control samples lacking FITC conjugate. The control values were then subtracted from the sample values.

Polarization data were analyzed according to the Perrin equation (Perrin, 1926)

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + \tau_F kT/Vn) \qquad (1)$$

where P is the sample polarization, P_0 the limiting polarization, τ_F the fluorescence lifetime, k the Boltzmann constant, T the temperature, n the viscosity, and V the molecular volume. According to the above equation, a plot of $1/P - 1/3$ vs. τ/n should be a straight line with the ordinate intercept being $1/P_0 - 1/3$. The slope is $(1/P_0 - 1/3)\tau_F k/V$ from which the molecular volume, V, is calculated by using the P_0 determined by the intercept and the τ_F determined independently. Viscosity was adjusted by varying the sucrose concentration while maintaining the temperature at 25 °C. Viscosity values for the various sucrose concentrations were obtained from the literature (Sobier, 1970). Lines were fitted to the data by using least-squares linear regression analysis. Lifetime measurements were made by using both frequency phase and frequency modulation data obtained on an SIM 4800 subnanosecond phase shift fluorometer, and the average value of the two methods was reported. The data were not corrected for the wavelength-dependent time response of the photomultiplier. Lifetime analysis at a single frequency was performed in the presence and absence of 10 mM Ca^{2+}.

[1] Abbreviations: FITC, fluorescein isothiocyanate; cmc, critical micelle concentration; DPC, dodecylphosphocholine; diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; diacyl-PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PLA, phospholipase A; PLA_2, phospholipase A_2.

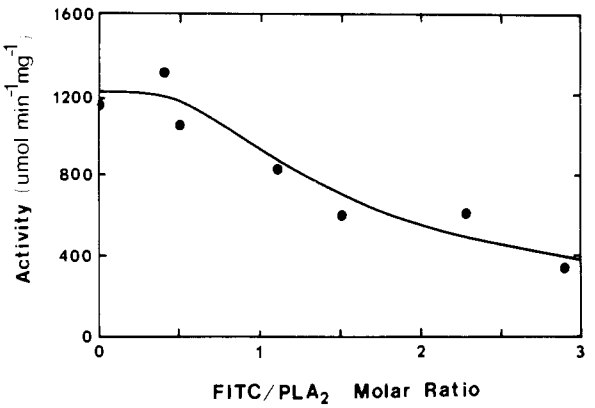

FIGURE 1: Activity of *Naj naja naja* phospholipase A_2 as a function of bound fluorescein isothiocyanate.

The molecular weight estimates, assuming globular shape, were obtained from

$$M_r = VN/\bar{v} \quad (2)$$

where M_r is the relative molecular weight, V is the molecular volume, $\bar{v}$ is the specific volume, and N is Avogadro's number. Specific volume values used for the *C. adamanteus* and the pancreatic enzymes were 0.72 (Wells & Hanahan, 1969) and 0.71 $cm^3\ g^{-1}$ (Hille et al., 1981), respectively. A value of 0.70 $cm^3\ g^{-1}$ for the *N. naja naja* phospholipase was calculated from the amino acid composition (Darke et al., 1980) by the technique of McMeekin et al. (1949).

Quenching Studies. Quenching experiments were performed by adding a concentrated NaI solution to an enzyme conjugate solution and measuring the fluorescence intensity after each addition, correcting for the dilution. Sodium thiosulfate (10 mM) served to keep I^- in the reduced form. The modified Stern–Volmer plot (Lehrer, 1971), given in eq 3, was used to

$$F_0/\Delta F = 1/f_a K[Q] + 1/f_a \quad (3)$$

analyze the data. F_0 is the fluorescence intensity in the absence of quenching, ΔF is the difference between F_0 and the intensity in the presence of quencher, f_a is the fraction of accessible fluorophore, K is the quenching constant, and [Q] is the concentration of quencher. In plots of $F_0/\Delta F$ vs. $[I^-]^{-1}$, the ordinate intercept gives the reciprocal of the fraction of total fluorophore that is accessible, and the slope gives the reciprocal of the accessible fluorophore and the quenching constant K.

RESULTS

FITC Coupling. A plot of activity as a function of bound FITC is shown in Figure 1. As the amount of bound FITC was increased, the activity of the *N. naja naja* decreased. FITC is known to modify free amino groups, and at approximately 3 mol of FITC bound per mole of phospholipase, 30% of the original activity remained. It has been shown that this phospholipase can be modified with up to 3.2 mol of trinitrobenzenesulfonate per mole of enzyme with 64% of the activity remaining (P. Darke and E. A. Dennis, unpublished results). Presumably, FITC modifies these same nonessential lysines. The *C. adamanteus* phospholipase A_2 shows a significant loss of activity when coupled to FITC. This can be explained by a previous study of Wells (1973) which demonstrated that the modification of a highly reactive lysine on the *C. adamanteus* phospholipase inhibited the enzyme. The pancreatic phospholipase A_2 showed the greatest loss of activity when coupled. This result is not unexpected since the pancreatic enzyme loses activity when the accessible N-terminal amino acid is modified (Slotboom & de Haas, 1975).

Table I: Polarization and Activity of Fluorescein–Phospholipase Conjugates

phospholipase[a]	FITC/PLA molar ratio	act. (%)	P_0	P	mol wt expt	mol wt lit.
N. naja naja	0.20	106	0.332	0.194	13000	13000
pancreas (porcine)	0.44	36	0.334	0.181	11000	14000
C. adamanteus	0.34	55	0.333	0.240	24000	30000

[a] Polarization measurements were in the presence of 15 mM Tris-HCl, pH 8.0, 100 nM NaCl, and 1 mM EDTA with 2.2, 3.6, or 3.1 $\mu g\ mL^{-1}$ samples of the pancreas, *Naja naja naja*, or *C. adamanteus* phospholipase A_2, respectively.

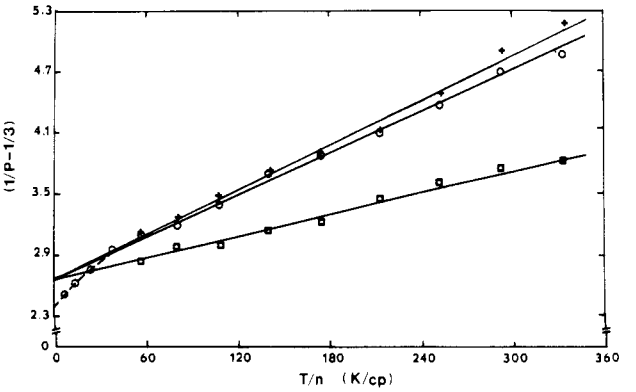

FIGURE 2: Perrin plots for *Naja naja naja* phospholipase A_2 (3.6 $\mu g\ mL^{-1}$) (○), *C. adamanteus* phospholipase A_2 (3.1 $\mu g\ mL^{-1}$) (□), and pancreas phospholipase A_2 (2.2 $\mu g\ mL^{-1}$) (+). Samples contained 15 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA. Viscosity was varied with sucrose (0–40%).

In the experiments reported here, only the conjugates containing less than 0.5 mol of FITC per mole of enzyme were used to assure that no more than one FITC was bound to any single phospholipase monomer. This was done to minimize self-energy transfer between two closely bound FITC groups. In addition, the *N. naja naja* phospholipase retained 100% of its activity at this level of modification. Table I shows the parameters calculated for the three FITC-coupled phospholipases.

Molecular Weight Determination. Fluorescence polarization was used to determine the apparent molecular weight of the enzyme. The polarization (P) and the limiting polarization (P_0) for all three of the enzyme–FITC conjugates are given in Table I. The corresponding Perrin plots are shown in Figure 2. The linear region at high T/n values was used to extrapolate the limiting polarizations which are involved with macromolecular motion (Lakowicz, 1983). The downward curvature in the Perrin Plots below $T/n = 60$, as shown by a dashed line for the *N. naja naja* conjugate (Figure 2), represents structural motion of the probe and was not used for extrapolation of the limiting polarization. The experimentally calculated molecular weights and the literature values are shown. The molecular weights calculated from the Perrin equation were similar to the literature values for all three enzyme–FITC conjugates (Table I).

Effect of Increased Enzyme Concentration on Polarization. The phospholipase A_2 concentration-dependent aggregation was detected by changes in the fluorescence polarization. The polarization increased as unlabeled *N. naja naja* phospholipase A_2 was added to a fixed concentration of conjugated enzyme (Figure 3). The limiting polarization was redetermined at a phospholipase concentration of 0.5 mg mL^{-1} and had not changed. The extrapolated P_0 and the calculated molecular

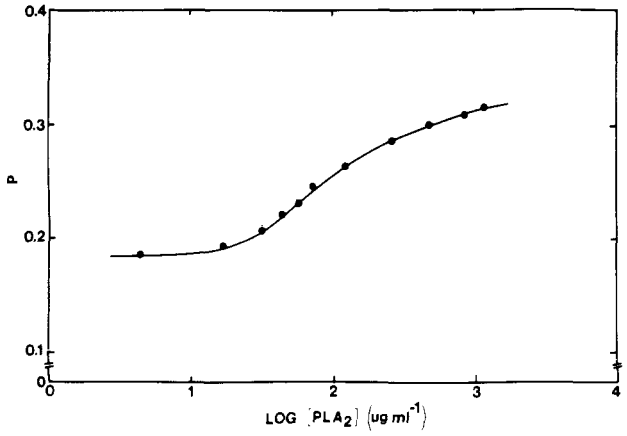

FIGURE 3: Polarization of *Naja naja naja* phospholipase A_2 as a function of concentration. Each sample contained 4.5 μg mL^{-1} phospholipase–fluorescein conjugate to which free phospholipase was added to achieve the final concentration shown. Each sample contained 15 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

Table II: Polarization Parameters of *Naja naja naja*: Fluorescein Conjugate under Various Conditions[a]

additions[a]	P_0	P	mol wt
none[b]	0.332	0.192	13 000
PLA_2 (0.5 mg mL^{-1})[c]	0.331	0.301	97 000
DPC (0.1 mM)[c]	0.356	0.287	40 000
diC_7PC (2 mM)[e]	0.350	0.248	23 000
Ca^{2+} (10 mM)[d]	0.356	0.281	36 000

[a] Each sample, unless specified, contained 15 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 3.6 μg mL^{-1} *Naja naja naja*–fluorescein conjugates. [b] Included 100 mM NaCl. [c] Contained 4.5 μg mL^{-1} *Naja naja naja*–fluorescein conjugate. [d] Contained 16 μg mL^{-1} *Naja naja naja*–fluorescein conjugate. [e] Diheptanoylphosphatidylcholine.

Table III: Effect of Divalent Metal Ions on the Polarization of the Phospholipase–Fluorescein Conjugate[a]

	P		
metal ion (1 mM)	*Naja naja naja* PLA_2	pancreatic PLA_2	*C. adamanteus* PLA_2
EDTA (1 mM)	0.197	0.185	0.236
Ca^{2+}	0.277	0.204	0.234
Ba^{2+}	0.242	0.203	0.232
Mn^{2+}	0.214	0.196	0.231
Co^{2+}	0.211	0.198	0.242
Mg^{2+}	0.198	0.193	0.232

[a] The buffer system contained 15 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 50 mM EDTA, except where a higher EDTA concentration is shown. The standard error for the data in this table was ±1.4% (triplicate samples).

weight under these conditions as well as in the presence of various additives are given in Table II.

Effect of Divalent Metal Ions on Polarization. A number of divalent metal ions were tested to determine if they influenced the shape or aggregation state of the FITC conjugates (Table III). Of those tested, only Ba^{2+} and Ca^{2+} showed any significant effect on the measured polarization, and then only with the *N. naja naja* phospholipase A_2 conjugate. The effect of Ca^{2+} concentration on the polarization of the *N. naja naja* conjugate is shown in Figure 4. The half-maximal effect occurred at a Ca^{2+} concentration of 150 μM. In the presence of 10 mM Ca^{2+}, the conjugate was found to have a higher limiting polarization, as extrapolated from Figure 5; the fluorescence lifetime was 2.75 ns in the absence of Ca^{2+} and 2.6 ns in the presence of Ca^{2+}, which confirms that the polarization changes are not caused by changes in the fluorescence lifetime to 2.61 ns. The limiting polarizations and

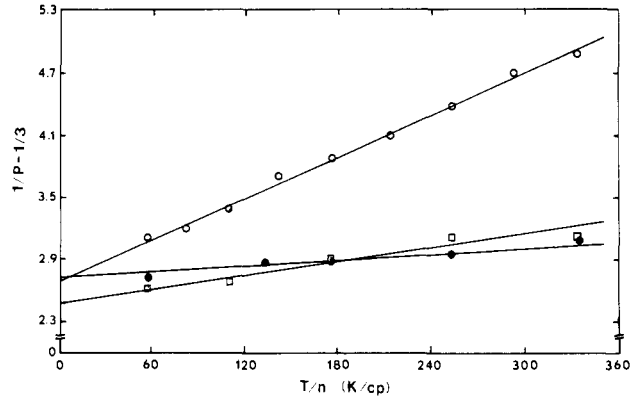

FIGURE 4: Perrin plots of 3.6 μg mL^{-1} *Naja naja naja* phospholipase A_2 conjugate (○) in the presence of 10 mM $CaCl_2$ (□) or 0.5 mg mL^{-1} unlabeled phospholipase (●). Each sample contained 100 mM NaCl (omitted for the 0.5 mg mL^{-1} phospholipase sample), 15 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

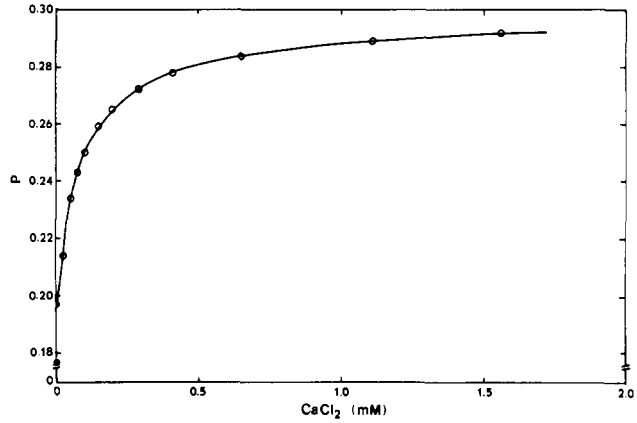

FIGURE 5: Effect of $CaCl_2$ on the polarization of the *Naja naja naja* phospholipase–fluorescein conjugate. The sample contained 100 mM NaCl, 15 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 16 μg mL^{-1} conjugate. The standard error for the data was ±1.5% for triplicate samples.

apparent molecular weights are given in Table II.

Effects of Lipids on Polarization. The polarization was determined in the presence of diheptanoyl-PC and dodecylphosphocholine. Dodecylphosphocholine is a nonhydrolyzable phospholipid analogue containing a 12 carbon chain with a phosphocholine head group. It is an excellent activator of the cobra venom enzyme (Plückthun & Dennis, 1982) with a cmc of 1.1 mM (Van Dam-Mieras et al., 1975). The diheptanoyl-PC is a hydrolyzable synthetic short-chain lecithin substrate with a cmc of 1.6 mM (Van Eijk et al., 1983) which conveniently allows one to study both monomeric and micellar forms. Concentrations of dodecylphosphocholine between 0 and 90 μM caused an increase in the polarization of *N. naja naja* conjugate (Figure 6). The polarization increased well beyond that found for the *C. adamanteus* dimer, suggesting that the cobra venom enzyme undergoes a more extensive aggregation. The P_0 and the calculated molecular weight for a saturating dodecylphosphocholine concentration are given in Table II. In the presence of increasing diheptanoyl-PC, the *N. naja naja* phospholipase showed a biphasic increase in polarization (Figure 7). An increase below 0.6 mM and another between 1.0 and 1.6 mM were observed. The polarization did not increase significantly above that found for the *C. adamanteus* enzyme and thus may in fact represent dimer formation. At 2 mM diheptanoyl-PC, the calculated molecular weight was 23 000 (Table II) which supports this contention.

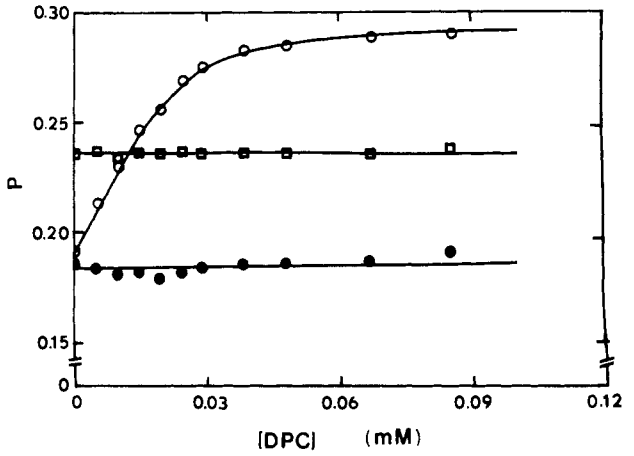

FIGURE 6: Polarization as a function of dodecylphosphocholine (DPC) concentration. A fluorescein conjugate of each of the three phospholipases was tested. Concentrations were 3.6, 3.1, and 3.0 $\mu g\ mL^{-1}$ for *Naja naja naja* phospholipase A_2 (○), *C. adamanteus* phospholipase A_2 (□), and pancreatic phospholipase A_2 (●), respectively. Samples contained 100 mM NaCl, 15 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

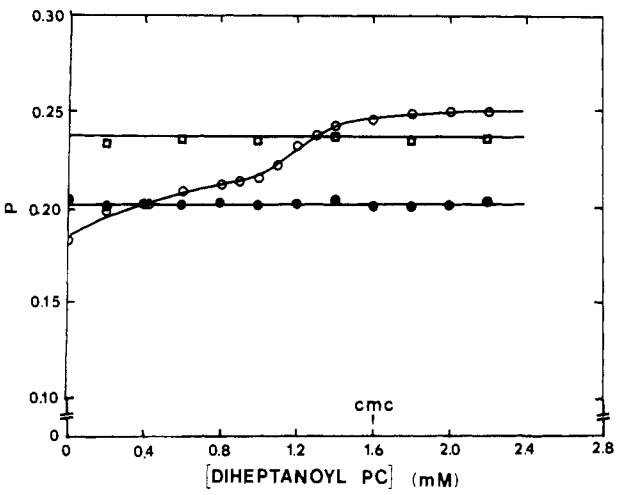

FIGURE 7: Polarization as a function of diheptanoyl-PC concentration. Measurements were for 3.6 $\mu g\ mL^{-1}$ *Naja naja naja* phospholipase A_2 (○), 3.1 $\mu g\ mL^{-1}$ *C. adamanteus* phospholipase A_2 (□), and 2.2 $\mu g\ mL^{-1}$ porcine pancreatic phospholipase A_2 (●). All samples contained 15 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The reported cmc of this phospholipid is indicated.

Quenching Experiments. Quenching was performed with I^-, a commonly used collisional quencher (Lehrer, 1971). The concentration of quencher was increased in samples containing *N. naja naja* conjugate in the presence or absence of Ca^{2+} or added unlabeled enzyme. The modified Stern–Volmer plot gave a linear dependence of the quenching data (Figure 8). The increased slope in the presence of 0.5 mg mL^{-1} unlabeled *N. naja naja* phospholipase was suggestive of decreasing accessibility of I^- to bound FITC. In the presence of Ca^{2+}, the change in the slope was less significant.

Discussion

The *N. naja naja* phospholipase A_2 catalyzed hydrolysis of phosphatidylethanolamine (PE) can be activated by phosphocholine-containing lipids. This activation and related experiments indicate that the binding of two phospholipids is important for catalysis (Roberts et al., 1979; Adamich et al., 1979; Plückthun & Dennis, 1982). Two models have been proposed to explain these results, one involving a monomer with two phospholipid binding sites and the other a dimer with one lipid binding site per subunit (Dennis, 1983). Efforts have been made to determine the aggregation state of the *N. naja naja* enzyme. It appears to be a monomer, although at enzyme concentrations above 30 $\mu g\ mL^{-1}$ it aggregates (Deems & Dennis, 1975). All previous attemps to directly determine the aggregation state of the enzyme in the presence of Ca^{2+} and substrate have been hindered by experimental limitations at the very low enzyme concentrations required.

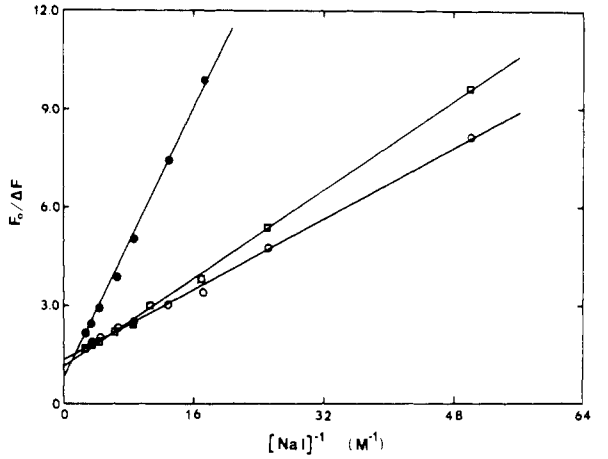

FIGURE 8: Modified Stern–Volmer quenching plot of 4.5 $\mu g\ mL^{-1}$ *Naja naja naja* phospholipase A_2 conjugate (○) in the presence of 10 mM $CaCl_2$ (3.6 $\mu g\ mL^{-1}$ conjugate) (□) or in the presence of 0.5 mg mL^{-1} added phospholipase (4.5 $\mu g\ mL^{-1}$ conjugate) (●). Each sample contained 100 mM NaCl, 15 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10 mM sodium thiosulfate.

When a chemically modified enzyme is studied, a prime concern is whether the labeled enzyme behaves as its unlabeled counterpart. The *N. naja naja* conjugate used throughout this work was fully active, indicating that at least kinetically the conjugate and native enzymes are similar. The native *N. naja naja* phospholipase is known to undergo a concentration-dependent aggregation (Deems & Dennis, 1975; Lewis et al., 1977) and has been observed to aggregate in the presence of dodecylphosphocholine (Plückthun & Dennis, 1985). Both of these observations were reproduced by using fluorescence polarization with the labeled enzyme. Additionally, the quenching experiment demonstrates that an increase in the phospholipase A_2 concentration decreases probe accessibility, suggesting protein aggregation. That the modified enzyme maintains full activity and undergoes concentration- and dodecylphosphocholine-dependent aggregation clearly indicates that the *N. naja naja* conjugate acts like the native, unlabeled phospholipase.

Another important question in polarization experiments is whether the probe accurately reports the motion of the macromolecular and whether this motion can be used to determined the size of the macromolecule. To test the validity of such calculations and the assumptions on which they are based, the molecular weights derived from the polarization data were compared to those found in the literature. The *C. adamanteus* and pancreatic enzymes were calculated to have apparent molecular weights of 24 000 and 11 000, respectively. These compare well to the molecular weights of 30 000 and 14 000 in the literature. The apparent molecular weight for the *N. naja naja* phospholipase was 13 000, the same value as that estimated from the amino acid analysis (Darke et al., 1980). It is clear that the polarization results not only correctly follow the trends in aggregation of the *N. naja naja* phospholipase but also accurately report the apparent molecular weights for the three labeled enzymes.

Having established that the polarization measurements on the FITC–enzyme conjugate were reporting the aggregation

state of the native enzyme, we then used this conjugate to determine the aggregation state of the phospholipase A_2 in the presence of either substrate or cofactor. The addition of Ca^{2+} alone caused an aggregation of the enzyme. The effect did not appear to be due to an interaction between Ca^{2+} and the label since neither the pancreatic nor the *C. adamanteus* conjugates demonstrated this behavior. The effect appeared to be specific for Ca^{2+} since other divalent metals, with the exception of Ba^{2+}, had no effect. Ba^{2+} is known to be a competitive inhibitor of Ca^{2+} (Roberts et al., 1977b; Dennis et al., 1981; Van Dam-Mieras et al., 1975) and would be expected to cause similar changes as found. If the change in aggregation is due to binding at the active site, then the concentration dependence of this effect should be similar to the enzyme's Ca^{2+} binding constant. Indeed, the half-maximal polarization change was found to occur at the same concentration as the reported Ca^{2+} binding constant of 0.15 mM (Roberts et al., 1977b). Since the Ca^{2+} binding characteristics of aggregation are similar to those of the native enzyme, it seems likely that the aggregation is caused by Ca^{2+} binding in the catalytic site and not some nonspecific Ca^{2+}–protein interaction. If aggregation occurs, the accessibility of the probe to solube quencher may decrease, and this was observed in the presence of Ca^{2+} although not to the extent observed in the presence of additional phospholipase.

The substrate diheptanoyl-PC causes aggregation of the *N. naja naja* conjugate below the cmc but does not increase the aggregation above it. This suggests that lipid monomers were responsible for the aggregation effect and not lipid micelles. In the present study, dodecylphosphocholine was also found to cause aggregation below its cmc. Presumably, this is due to enzyme aggregation, though it may be that lipid–protein micelles are formed. If the protein were part of such a structure, the fluorescence polarization would be indicating the micelle molecular weight and not the true molecular weight of the protein or protein aggregate. The formation of such micelles seems unlikely since the calculated molecular weight of 23 000 is much lower than one would expect for a micelle, and the lipid concentrations used were below the cmc. In the presence of micellar substrate analogues, the monomeric pancreatic enzyme was reported by de Haas and co-workers (Soares de Araujo et al., 1979; Hille et al., 1981) to form a complex containing more than one protein molecule. However, these investigators interpreted their data in terms lipid–protein complexes and explicitly pointed out that this was not evidence for lipid-induced aggregation of phospholipase A_2 to an active dimer as we (Roberts et al., 1977a) originally proposed. Other experiments on the *N. naja naja* enzyme by us (Plückthun, 1982; Plückthun & Dennis, 1985) and on the *Naja melanoleucca* enzyme by Van *eijk* et al. (1983) now show that lipid–protein complexes containing more than one protein molecule are induced at monomeric concentrations of substrate analogues.

Activation of the *N. naja naja* phospholipase A_2 toward PE requires the presence of an interface (Plückthun et al., 1985) while the aggregation effects presented here do not require an interface. Therefore, aggregation alone is not sufficient to activate the enzyme. Apparently, a combination of aggregation and interfacial interactions is required to fully activate the enzyme.

There does seem to be a difference in the effects of the binding of Ca^{2+} and the binding of lipid monomers. Enzyme aggregated due to monomeric lipid binding does not bind to micelles as indicated by a constant polarization through and above the cmc. Gel filtration experiments suggest that in the presence of Ba^{2+} or Ca^{2+} micelle binding does occur (Roberts et al., 1977a,b). Either the aggregation state induced by monomer phospholipid is not the same as that caused by Ca^{2+} or, if the aggregation states are similar, the aggregated enzyme still requires Ca^{2+} to bind to the micelle. It is difficult to see how Ca^{2+} would affect the binding to an interface without affecting the binding of monomeric PC. Presumably, the two aggregation states are different.

These results clearly indicate that in the presence of Ca^{2+} or in the presence of monomeric phosphocholine-containing lipids, the *N. naja naja* phospholipase aggregates. It is difficult to imagine that under assay conditions where both substrate and Ca^{2+} are present, the enzyme would not be aggregated. Thus, the conclusion is that the enzyme is in an aggregated state under assay conditions. This supports the dual phospholipid model proposed for the mechanism of action of the *N. naja naja* phospholipase A_2 (Roberts et al., 1977a; Dennis et al., 1981).

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Registry No. Ca, 7440-70-2; Ba, 7440-39-3; PLA_2, 9001-84-7; DPC, 29557-51-5; DiC_7PC, 35387-75-8.

REFERENCES

Adamich, M., Roberts, M. F., & Dennis, E. A. (1979) *Biochemistry 18*, 3308–3314.
Chryssomallis, G. S., Torgerson, P. M., Drickamer, H. G., & Weber, G. (1981) *Biochemistry 20*, 3955–3959.
Darke, P. L., Jarvis, A. A., Deems, R. A., & Dennis, E. A. (1980) *Biochim. Biophys. Acta 626*, 154–161.
Deems, R. A., & Dennis, E. A. (1975) *J. Biol. Chem. 250*, 9008–9012.
Deems, R. A., & Dennis, E. A. (1981) *Methods Enzymol. 71*, 703–710.
Dennis, E. A. (1973) *J. Lipid Res. 14*, 152–159.
Dennis, E. A. (1983) *Enzymes, 3rd Ed. 16*, 307–353.
Dennis, E. A., Darke, P. L., Deems, R. A., Kensil, C. R., & Plückthun, A. (1981) *Mol. Cell. Biochem. 36*, 37–45.
Hazlett, T. L., & Dennis, E. A. (1985) *Toxicon 23*, 457–466.
Hille, J. D. R., Donne-Op den Kelder, G. M., Sauve, P., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4068–4073.
Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
Lewis, R. V., Roberts, M. F., Dennis, E. A., & Allison, W. S. (1977) *Biochemistry 16*, 5650–5654.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
McMeekin, T. L., Groves, M. L., & Hipp, N. J. (1949) *J. Am. Chem. Soc. 71*, 3298–3300.
Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32*, 147–154.
Perrin, F. (1926) *J. Phys. Radium 7*, 390–401.
Plückthun, A. (1982) Ph.D. Thesis, University of California, San Diego.

Plückthun, A., & Dennis, E. A. (1982) *Biochemistry 21*, 1750-1756.
Plückthun, A., & Dennis, E. A. (1985) *J. Biol. Chem.* (in press).
Plückthun, A., Rohlfs, R., Davidson, F., & Dennis, E. A. (1985) *Biochemistry 24*, 4201-4208.
Roberts, M. F., Deems, R. A., & Dennis, E. A. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1950-1954.
Roberts, M. F., Deems, R. A., & Dennis, E. A. (1977b) *J. Biol. Chem. 252*, 6011-6017.
Roberts, M. F., Adamich, M., Robson, R. J., & Dennis, E. A. (1979) *Biochemistry 18*, 3301-3308.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53-56.
Slotboom, A. J., & de Haas, G. H. (1975) *Biochemistry 14*, 5394-5399.
Smith, C. M., & Wells, M. A. (1981) *Biochim. Biophys. Acta 663*, 687-694.
Soares de Araujo, P., Rosseneu, M. Y., Kremer, J. M. H., Van Zoelen, E. J. J., & de Haas, G. H. (1979) *Biochemistry 18*, 580-585.
Sobier, H. A. (1970) *Handbook of Biochemistry Selected Data for Molecular Biology*, pp J 288-J 291, Chemical Rubber Publishing Co., Cleveland, OH.
Tengerdy, R. P., & Chang, C. (1966) *Anal. Biochem. 16*, 377-383.
Van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387-5394.
Van Eijk, J. H., Verheij, H. M., Dijkman, R., & de Haas, G. H. (1983) *Eur. J. Biochem. 132*, 183-188.
Weber, G. (1952a) *Biochem. J. 51*, 145-155.
Weber, G. (1952b) *Biochem. J. 51*, 155-167.
Wells, M. A. (1971) *Biochemistry 10*, 4073-4083.
Wells, M. A. (1973) *Biochemistry 12*, 1086-1093.
Wells, M. A., & Hanahan, D. J. (1969) *Biochemistry 8*, 414-424.

Peroxidase-Catalyzed N-Demethylation Reactions: Deuterium Solvent Isotope Effects†

Gregory L. Kedderis‡ and Paul F. Hollenberg*
Departments of Pathology, Molecular Biology, and Biochemistry and the Cancer Center, Northwestern University Medical School, Chicago, Illinois 60611

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ABSTRACT: The effect of D_2O on the kinetic parameters for the hydroperoxide-supported N-demethylation of *N,N*-dimethylaniline catalyzed by chloroperoxidase and horseradish peroxidase was investigated in order to assess the roles of exchangeable hydrogens in the demethylation reaction. The initial rate of the chloroperoxidase-catalyzed N-demethylation of *N,N*-dimethylaniline supported by ethyl hydroperoxide exhibited a pL optimum (where L denotes H or D) of 4.5 in both H_2O and D_2O. The solvent isotope effect on the initial rate of the chloroperoxidase-catalyzed demethylation reaction was independent of pL, suggesting that the solvent isotope effect is not due to a change in the pK of a rate-controlling ionization in D_2O. The solvent isotope effect on the V_{max} for the chloroperoxidase-catalyzed demethylation reaction was 3.66 ± 0.62. In contrast, the solvent isotope effect on the V_{max} for the horseradish peroxidase catalyzed demethylation reaction was approximately 1.5 with either ethyl hydroperoxide or hydrogen peroxide as the oxidant, indicating that the exchange of hydrogens in the enzyme and hydroperoxide for deuterium in D_2O has little effect on the rate of the demethylation reaction. The solvent isotope effect on the V_{max}/K_M for ethyl hydroperoxide in the chloroperoxidase-catalyzed demethylation reaction was 8.82 ± 1.57, indicating that the rate of chloroperoxidase compound I formation is substantially decreased in D_2O. This isotope effect is suggested to arise from deuterium exchange of the hydroperoxide hydrogen and of active-site residues involved in compound I formation. A solvent isotope effect of 2.96 ± 0.57 was observed on the V_{max}/K_M for *N,N*-dimethylaniline in the chloroperoxidase-catalyzed reaction. This isotope effect is suggested to arise from deuterium exchange of the α-carbon hydrogens of the anilinium cation radical intermediate in the chloroperoxidase-catalyzed demethylation reaction.

Chloroperoxidase (chloride:hydrogen peroxide oxidoreductase; EC 1.11.1.10) and horseradish peroxidase (donor:hydrogen peroxide oxidoreductase; EC 1.11.1.7) catalyze the hydroperoxide-supported N-demethylation of a variety of *N*-methylarylamine compounds (Kedderis et al., 1980; Kedderis & Hollenberg, 1983a). The results of a steady-state kinetic analysis of the chloroperoxidase-catalyzed N-demethylation of *N,N*-dimethylaniline (DMA)[1] supported by ethyl hydroperoxide (EtOOH) were consistent with a ping-pong Bi-Bi kinetic mechanism for the reaction (Kedderis & Hollenberg, 1983b). Initial velocity studies of the horseradish peroxidase catalyzed demethylation of DMA were also consistent with a ping-pong mechanism (Kedderis & Hollenberg, 1983a). In this mechanism, the hydroperoxide reacts with the native peroxidase to form the oxidized enzyme intermediate

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* Address correspondence to this author at the Department of Pathology, Northwestern University Medical School.

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[1] Abbreviations: DMA, *N,N*-dimethylaniline; EtOOH, ethyl hydroperoxide.

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