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Identification of a Membrane-Bound, Glycol-Stimulated Phospholipase A_2 Located in the Secretory Granules of the Adrenal Medulla†

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ABSTRACT: Chromaffin granule membranes prepared from bovine adrenal medullae showed Ca^{2+}-stimulated phospholipase A_2 (PLA_2) activity when assayed at pH 9.0 with phosphatidylcholine containing an [^{14}C]-arachidonyl group in the 2-position. However, the activity occurred in both soluble and particulate subcellular fractions, and did not codistribute with markers for the secretory granule. PLA_2 activity in the granule membrane preparation was stimulated dramatically by addition of glycerol, ethylene glycol, or poly(ethylene glycol). This glycol-stimulated PLA_2 activity codistributed with membrane-bound dopamine β-hydroxylase, a marker for the granule membranes, through the sequence of differential centrifugation steps employed to prepare the granule membrane fraction, as well as on a sucrose density gradient which resolved the granules from mitochondria, lysosomes, and plasma membrane. The glycol-stimulated PLA_2 of the chromaffin granule was membrane-bound, exhibited a pH optimum of 7.8, retained activity in the presence of EDTA, and was inactivated by *p*-bromophenacyl bromide. When different ^{14}C-labeled phospholipids were incorporated into diarachidonylphosphatidylcholine liposomes, 1-palmitoyl-2-arachidonylphosphatidylcholine was a better substrate for this enzyme than 1-palmitoyl-2-oleylphosphatidylcholine or 1-acyl-2-arachidonyl-phosphatidylethanolamine, and distearoylphosphatidylcholine was not hydrolyzed.

The secretion of peptides, hormones, and most neurotransmitters occurs by a similar exocytotic mechanism in a variety of endocrine, exocrine, and neurosecretory cell types (Kelly, 1985), including the well-characterized chromaffin cells of the adrenal medulla (Burgoyne, 1984; Pollard et al., 1985; Strittmatter, 1988; Winkler, 1988). Stimulation of chromaffin cells leads to an increase in the intracellular Ca^{2+} concentration (Knight & Kesteven, 1983), which triggers secretion (Baker & Knight, 1978; Dunn & Holz, 1983; Wilson & Kirshner, 1983). Bilayer fusion between the secretory vesicle membrane and the plasmalemma is believed to represent a kinetically difficult step, and appears to be Ca^{2+}-dependent (Winkler, 1988; Plattner, 1989). The regulation of secretory vesicle transport, cytoskeletal rearrangements, and other requisite steps in the overall exocytotic process by Ca^{2+} or other factors has not yet been clearly defined (Gomperts, 1986; Sarafian et al., 1987; Aunis & Bader, 1988; Strittmatter, 1988; Holz et al., 1989).

A number of lines of evidence suggest the participation of a phospholipase A_2 (PLA_2)[1] in exocytosis in chromaffin and other neuroendocrine tissue. Enzymes in the PLA_2 family catalyze hydrolysis of the *sn*-2 acyl ester bonds in phospholipids, to release 1-acyllysophospholipid and free fatty acid. Phospholipids occurring in membranes of chromaffin cells, as in most cells, have an acyl chain composition such that the fatty acids liberated by PLA_2 action consist primarily of arachidonic acid and other cis-unsaturated fatty acids (Winkler & Smith, 1968; Balzer & Khan, 1975). The important roles of arachidonic acid in cellular signalling are well recognized, acting both as a precursor in eicosanoid biosynthesis and as

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[1] Abbreviations: PLA_2, phospholipase A_2; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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a direct modulator of protein kinase C, Ca^{2+} flux, and other processes (Axelrod et al., 1988; Smith, 1989). The cis-unsaturated fatty acids were shown to facilitate synthetic liposome and protein-mediated chromaffin granule fusion reactions in vitro (Meers et al., 1988; Creutz, 1981). Lysophospholipids have also been demonstrated to act as fusogens in vitro, through direct perturbation of bilayer structure (Poole et al., 1970; Cullis & Hope, 1978). Thus, both classes of products of the PLA_2 reaction may participate in the membrane fusion step. Indeed, treatment of plasma membrane vesicles with exogenous PLA_2 enhanced their ability to fuse with chromaffin granule membranes (Karli et al., 1990).

Further evidence for involvement of arachidonate in exocytosis comes from whole-cell secretion studies. Application of exogenous cis-unsaturated fatty acids enhanced catecholamine secretion in response to acetylcholine in intact chromaffin cells (Rindlisbacher et al., 1990), or Ca^{2+}-induced secretion by permeabilized cells (Koda et al., 1989). Frye and Holz (1984, 1985) found that release of arachidonic acid from cellular phospholipids accompanied secretion by stimulated cells.

While the data to suggest arachidonate involvement in exocytosis are persuasive, the metabolic pathway for its formation has not been clearly established. In bovine chromaffin cells, muscarinic stimulation causes phosphoinositide turnover without evoking secretion, whereas nicotinic stimulation leads to catecholamine secretion in the absence of phosphoinositide turnover (Fisher et al., 1981). These findings suggest that secretion may be coupled to free arachidonic acid production by another route in addition to or in place of the phospholipase C/diglyceride lipase pathway. In support of the hypothesis that phospholipase A_2 may be involved, both arachidonate release and catecholamine secretion were blocked when chromaffin cells were pretreated with *p*-bromophenacyl bromide, a nonspecific inhibitor of PLA_2 (Frye & Holz, 1985). However, little is known about the regulation or subcellular distribution of PLA_2 in chromaffin tissue. Only acid-active forms of the enzyme had been demonstrated in lysosomal fractions (Smith & Winkler, 1968; Bartolf & Franson, 1984), while others have not been able to measure any PLA_2 activity in chromaffin plasma membrane preparations (Zahler et al., 1986). The lipid composition of the membranes of the chromaffin granules is unusual, in that high levels of lysophospholipids and of long-chain free fatty acids are present (Blaschko et al., 1967; Frischenschlager et al., 1983; Husebye & Flatmark, 1984). On the basis of these observations, the presence of PLA_2 in the chromaffin granules would be anticipated. Indeed, purified chromaffin granule membrane preparations were demonstrated to contain PLA_2 activity which hydrolyzed either exogenous or endogenous phospholipid (Husebye & Flatmark, 1987). Although these authors did not investigate the distribution of this activity in different subcellular fractions, it could be distinguished from the lysosomal enzymes by its alkaline pH optimum. With respect to exocytotic mechanisms, a granule-specific PLA_2 would be particularly significant for its potential role in the in situ generation of fusogenic metabolites that may promote interaction between the secretory granule and the plasma membrane.

In the present report, we have undertaken a more thorough analysis of the subcellular compartmentation of PLA_2 activity in bovine chromaffin tissue. Evidence is presented for the existence of multiple forms of the enzyme in soluble and organellar compartments, and for the occurrence of at least one membrane-bound PLA_2 that is specifically located in the chromaffin granules. Conditions have been found under which the activity of the granule membrane PLA_2 is highly stimulated, permitting this enzyme to be selectively assayed even in impure membrane preparations. The enzyme has a pH optimum of 7.8, does not require Ca^{2+}, and can be inactivated by *p*-bromophenacyl bromide. Portions of this work were presented in preliminary form (Hildebrandt & Albanesi, 1989).

Experimental Procedures

Preparation of Chromaffin Granule Membranes. All solutions for the granule membrane preparation were buffered with 15 mM Tris-HCl, pH 7.5, and procedures were conducted at 0–4 °C. Medullae dissected from bovine adrenal glands were homogenized in 5 mL/g of buffer containing 0.3 M sucrose and 0.1 M NaCl. The dense organelle fraction was isolated by differential centrifugation (Smith & Winkler, 1967). Chromaffin granules were purified by centrifugation through a cushion of 1.8 M sucrose (Schneider, 1972) containing 0.1 M NaCl. The pellets were lysed hypotonically by resuspension in a volume of buffer equivalent to the original homogenate volume, then frozen, and thawed. The granule lysate was then centrifuged at 40000*g* for 30 min. The membrane pellet was washed by resuspending and repelleting at 40000*g* for 30 min, first from 0.25 M KCl and then from buffer. Aliquots of the final chromaffin granule membrane pellet, suspended in buffer, were stored at −70°C. Subsequent dilutions of the preparation for addition to assays were made in buffer containing 0.3 M sucrose and 0.25 mM EDTA; if necessary, bovine serum albumin was included to maintain a protein concentration of at least 0.1 mg/mL.

Sucrose Gradient Fractionation. Portions (2.5 mL, 32 mg of protein) of the dense organelle fraction described above were applied to the top of the following step gradient: 5 mL each of 0.9, 1.2, 1.4, and 1.6 M sucrose; 7.5 mL of 1.8 M sucrose. The tubes were centrifuged in a swinging-bucket rotor at 100000*g*, 2 h. Fractions were collected from the gradient by using a peristaltic apparatus (Densi-flow IIc, Buchler Instruments, Fort Lee, NJ), lysed by freeze/thaw, and stored at −70 °C.

Marker Assays. Marker enzymes for identification of specific organelles in subcellular fractions were assayed under conditions such that product formation was proportional to protein concentration. Acetylcholinesterase was measured according to Ellman et al. (1961) and monoamine oxidase according to Wurtman and Axelrod (1963). Catecholamines were measured in supernatants from treatment of samples with 10% (w/v) trichloroacetic acid by a modification (Johnson & Scarpa, 1976) of the method of James (1948), using L-epinephrine as standard. Phenolphthalein glucuronide was used as substrate for the measurement of β-glucuronidase, and product was determined by comparison to a phenolphthalein standard curve (Fishman, 1974). Glucose-6-phosphatase was assayed as described (Baginski et al., 1974) with the following modifications: glucose phosphate concentration was 10 mM, the enzymatic reaction proceeded for 1 h, and the molybdate reaction was performed at 0 °C for 20 min. Membrane-bound dopamine β-hydroxylase was assayed in the presence of 0.04% Triton X-100 by the photometric procedure of Nagatsu (1985), using samples of membrane pellets prepared by lysis and ultracentrifugation, as described in the figure legends.

Protein was assayed (Lowry et al., 1951) after precipitation with 10% trichloroacetic acid (Cleland & Slater, 1953).

Phospholipase A_2 Assays. Phospholipase A_2 activity was measured in fixed-time incubations as the enzymatic release of free [^{14}C]arachidonic acid from 1-palmitoyl-2-[1-^{14}C]-

arachidonylphosphatidylcholine (52 Ci/mol; New England Nuclear, Wilmington, DE), incorporated into carrier liposomes of diarachidonyl-PC. The labeled and unlabeled phospholipids were mixed in $CHCl_3$/MeOH (2:1 v/v) at a mole ratio of (1–5):100, to give 25000 dpm per incubation and a total PC concentration of 0.05–0.1 mM in the final assay mixture. After evaporation of the solvent under N_2, the [^{14}C]PC mixture was sonicated into Tris buffer, pH 9.0, for 150 s/mL at a power setting of 12.5 using a microprobe sonicator (Model W-220F, Heat Systems-Ultrasonics, Long Island, NY).

Incubations of membrane suspensions with the [^{14}C]PC liposomal substrate were conducted at 37 °C, under the conditions specified in figure legends. Four volumes of $CHCl_3$/MeOH/acetic acid (9:4:1 v/v) were added to stop the enzymatic reaction and to extract the substrate and product. The $CHCl_3$ phases from glycol-containing samples were washed twice with 5–10 volumes of H_2O. Carrier amounts (20 μg of each) of arachidonic acid and diarachidonyl-PC were added to the lipid extracts, and samples could be conveniently stored in this state overnight at 4 °C. After evaporation under N_2, the lipids were taken up in $CHCl_3$/MeOH (2:1 v/v) and applied to polyester-backed silica thin-layer plates. The chromatographic solvent was *n*-heptane/diethyl ether/acetic acid (75:25:4 v/v) (Korte & Casey, 1982). The phosphatidylcholine (R_f = 0.01) and arachidonic acid (R_f = 0.4) spots were visualized with I_2 vapor and cut from the plate for scintillation counting.

Experiments included buffer blanks to determine extents of nonenzymatic hydrolysis, which were routinely less than 0.2%.

Results

Demonstration of Phospholipase A_2 Activity in Chromaffin Granule Membranes. For the initial assessment of PLA_2 activity in subcellular fractions of the adrenal medulla, we selected an assay pH of 9.0 in order to preferentially detect enzymes other than the acid-active phospholipases A_1 and A_2 that occur in lysosomes of many tissues including adrenal chromaffin (Smith & Winkler, 1968). Phosphatidylcholine labeled in the *sn*-2-position with [^{14}C]arachidonate was chosen as substrate, for the purpose of detecting enzymes capable of releasing arachidonic and other cis-unsaturated fatty acids from cellular phospholipids. Phosphocholine was the preferred head group for the substrate because sonicates of this neutral phospholipid form lamellar structures which are stable over a broad range of pH and ionic compositions. Millimolar amounts of calcium were routinely included in these incubations for two reasons. First, many phospholipases are Ca^{2+}-dependent, and second, Ca^{2+} has been shown to promote the rapid fusion of chromaffin granule membranes with phosphatidylcholine liposomes (Hildebrandt & Albanesi, 1990) and certain other phospholipid vesicles (Bental et al., 1984).

Under the simple assay conditions chosen, chromaffin granule membranes prepared by standard procedures were found to contain phospholipase activity that catalyzed production of free ^{14}C-labeled arachidonic acid when the membranes were incubated with sonicated liposomes containing the labeled phosphatidylcholine. The PLA_2 activity remained in the granule membrane preparation through treatment with 0.25 M KCl and the subsequent washing steps, indicating that the enzyme was strongly membrane-bound. In this simple assay system, product formation was linear with time for a period of at least 2 h (data not shown). However, although the rate of reaction increased with increasing concentration of membranes added, the relationship was not strictly linear (Figure 1). Thus, it has not been possible to calculate the absolute specific activity of the enzyme in the preparations. Nevertheless, within a given experiment, we could make valid comparisons of the relative enrichment of PLA_2 among different subcellular fractions by keeping constant the amount of protein present in each sample assayed.

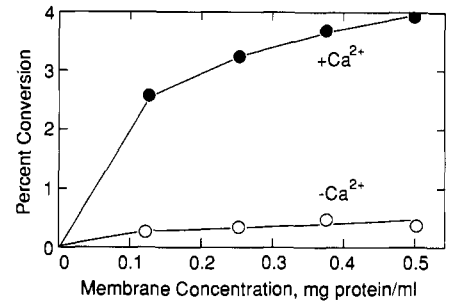

FIGURE 1: Chromaffin granule membranes catalyze hydrolysis of 1-palmitoyl-2-[^{14}C]arachidonylphosphatidylcholine. At time zero, granule membranes were added at the indicated protein concentrations to incubation mixtures containing 0.1 mM [^{14}C]PC liposomes, plus 0.3 M sucrose, 0.25 mM EDTA, and 50 mM Tris-HCl, pH 9.0, in the absence (○) or presence (●) of 2.5 mM $CaCl_2$. After 60 min, $CHCl_3$/MeOH/acetic acid was added to extract radioactive metabolites, which were separated by TLC and quantified. Product formation is expressed as percent of the labeled substrate that was converted to free arachidonate. The average of duplicate determinations is given for clarity.

Influence of Assay Conditions on Granule Membrane PLA_2 Activity. While certain intracellular PLA_2's have an absolute requirement for calcium, there are others which do not require Ca^{2+} for catalysis but can be stimulated by the metal ion (Brockerhoff & Jensen, 1974; van den Bosch, 1980). Under the conditions employed in Figure 1, the granule membrane PLA_2 activity appeared to be stimulated by $CaCl_2$. This effect was saturable, with half-maximal stimulation attained at 0.1–0.2 mM $CaCl_2$. In each of several independent granule membrane preparations, the stimulation induced by Ca^{2+} under these conditions fell in the range of 3–7-fold. However, this PLA_2 activity did not exhibit an absolute requirement for Ca^{2+}, since addition of 2 mM EDTA to the incubation did not significantly decrease the activity that was measured in the absence of added cation (data not shown).

Further experiments demonstrated that the activity of PLA_2 in granule membranes was strongly influenced by the composition of the assay buffer. Addition of NaCl to the incubations inhibited the activity to the low level measured in the absence of Ca^{2+}, with an I_{50} of about 10–50 mM. Similar effects were obtained with LiCl and KCl. These rather severe ionic strength effects made it unfeasible to determine the pH dependence of the activity because of the variable amounts of the counterion that would be introduced upon titration of the buffers.

Addition of up to 1.25 M sucrose to the incubation strongly enhanced PLA_2 activity measured in the presence of Ca^{2+} (Figure 2A). Because of the limited solubility of sucrose, higher concentrations could not be added to the assay. We therefore decided to test for stimulation of PLA_2 by other water-miscible liquid glycols, which could be used in the reaction mixture at high final concentrations. In the experiments shown in figure 2B–D, PLA_2 was measured in the presence or absence of Ca^{2+}, following the addition of glycerol, ethylene glycol, or poly(ethylene glycol) to the incubations. Each of these compounds resulted in profound stimulation of arachidonate release. The magnitudes of the stimulations attained at the optimal glycol concentrations were 32-fold in the presence of 60% glycerol plus 2.5 mM Ca^{2+}, 55-fold with 50% ethylene glycol plus EDTA, and 840-fold in the presence of

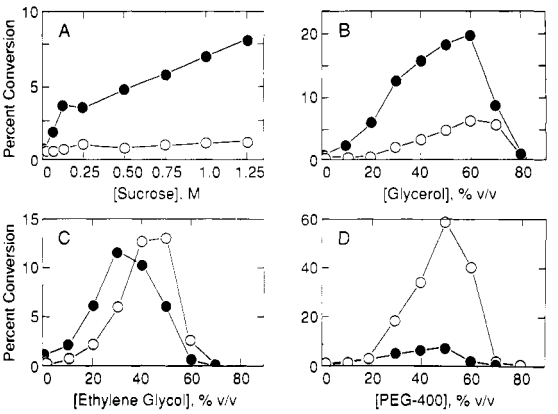

FIGURE 2: PLA_2 in chromaffin granule membranes is stimulated by glycol. Granule membranes (0.10 mg/mL for panel A or 0.06 mg/mL for panels B–D) were incubated with 0.1 mM [^{14}C]PC liposomes and the additions shown for 90 min (panel A), 30 min (panels B and D), or 40 min (panel C). All incubations contained 50 mM Tris-HCl, pH 9.0, 0.25 mM EDTA, and either 0 (○) or 2.5 mM (●) $CaCl_2$. The data shown are from single representative experiments which were reproducible.

50% PEG plus EDTA. These large effects made it necessary to reduce the incubation time and/or the protein concentrations in order to measure initial rates of product formation. Although poly(ethylene glycol) addition gave the highest activity, its use was inconvenient due to the tendency for nonvolatile material to carry over into the chloroform extracts, necessitating multiple washing steps prior to TLC. For routine experiments, the use of glycerol to stimulate PLA_2 activity was preferable.

Buffer blanks demonstrated that the increased formation of labeled product in the presence of these stimulating agents did not occur nonenzymatically. In additional control experiments, the glycol was added after the enzyme incubation was stopped. These experiments verified that the efficiencies of extraction of arachidonic acid and of phosphatidylcholine from the mixture into $CHCl_3$ were unaffected by the presence of glycerol, ethylene glycol, or poly(ethylene glycol) (data not shown). Hence, the compounds stimulated the rate of release of [^{14}C]arachidonic acid through enhancement of enzymatic activity in the preparation.

The influence of Ca^{2+} on PLA_2 activity under the glycol-stimulated conditions proved to be complex (Figure 2). At all concentrations of glycerol (Figure 2B), as with sucrose, activity was enhanced severalfold by addition of 2.5 mM $CaCl_2$. In contrast, Ca^{2+} was inhibitory over the entire range of concentrations of poly(ethylene glycol) (Figure 2D). Further, Ca^{2+} stimulated the activity at concentrations of ethylene glycol up to 30% but became inhibitory in the presence of 40% or greater ethylene glycol (Figure 2C). The mechanism for the stimulatory or inhibitory effects of Ca^{2+} on PLA_2 activity measured in the presence of glycols is presently uncertain. Regardless, it is clear that the presence of Ca^{2+} is not an absolute requirement for catalysis by the glycol-stimulated PLA_2.

Demonstration of the Absence of Radioactive Metabolites Other than Arachodonic Acid. In principle, release of labeled fatty acid from this substrate could occur not only by the direct action of PLA_2 but also by the sequential action of phospholipase C and diglyceride lipase, or by phospholipase A_1 and lysophospholipase, or by phospholipase D and PLA_2. Any of these alternative two-step lipolytic pathways would necessarily involve a labeled intermediate: diglyceride, lysophosphatidylcholine, or phosphatidic acid, respectively. The following chromatographic analysis demonstrated that the stimulation of arachidonate release observed with glycols resulted from changes in the activity of PLA_2 itself and was not caused by activation of these alternative pathways of phospholipid degradation. Chromaffin granule membranes were incubated with [^{14}C]PC liposomes (0.1 mM) under either stimulated (50% glycerol, 1 mM EDTA plus 5 mM $CaCl_2$, and 0.03 mg/mL membranes, 30 min) or nonstimulated (0.25 M sucrose, 1 mM EDTA, 5 mM $CaCl_2$, and 0.12 mg/mL membranes, 90 min) conditions. The lipid extracts containing labeled substrate and product (68 nCi per sample) were mixed with standards (diglyceride, monoglyceride, phosphatidic acid, phosphatidylcholine, lysophosphatidylcholine, and arachidonic acid) and separated by two-dimensional silica thin-layer chromatography in $CHCl_3$/MeOH/H_2O (63:27:4) followed by *n*-heptane/ethyl ether/acetic acid (75:25:4). After autoradiography of the plates for 10 days, the positions of radioactive spots were compared to the positions of standards visualized with I_2, with molybdenum blue, or by misting the plate with water. For all three assay conditions, significant radioactivity was found only in the phosphatidylcholine and arachidonic acid spots. Because radioactivity was not detectable in the spots corresponding to lysophosphatidylcholine, phosphatidic acid, diglyceride, or monoglyceride, the activation of phospholipase A_1, phospholipase D, phospholipase C, and diglyceride lipase in the granule membrane preparation was negligible under the conditions employed here to measure PLA_2 activity, including the glycol-stimulated conditions.

Sensitivity of PLA_2 Activity to Bromophenacyl Bromide. All the mammalian forms of PLA_2 that have been characterized to date can be irreversibly inactivated by the histidine-alkylating agent *p*-bromophenacyl bromide (Chang et al., 1987). The susceptibility of chromaffin granule membrane PLA_2 to inactivation by this reagent was investigated as follows. The membranes (0.5 mg/mL) were preincubated at 30 °C in 15 mM Tris-HCl (pH 7.5)/0.3 M sucrose/0.25 mM EDTA in the absence or presence of 2 μmol/mL bromophenacyl bromide. The effective concentration of the inhibitor is limited by its water solubility, about 0.1 mM (Volwerk et al., 1974). Samples from the preincubations were ultracentrifuged (Beckman airfuge, 25 psi, 20 min) to pellet the membranes, which were then assayed in duplicate for PLA_2 activity under the nonstimulated conditions (as in the legend to Figure 1; 0.38 mg/mL granule membranes and 2.5 mM $CaCl_2$, 60 min) or glycol–stimulated conditions (50% glycerol instead of sucrose, 0.033 mg/mL membranes, and 1 mM $CaCl_2$, 30 min). Treatment of the membranes for 24 h with bromophenacyl bromide resulted in complete inactivation of PLA_2, whether the activity was assayed with or without glycol stimulation. By comparison, there was 67 ± 6% recovery of the nonstimulated PLA_2 activity and 122 ± 7% recovery of glycol-stimulated activity from 24-h control preincubations without the inhibitor, demonstrating reasonable stability under the chosen conditions.

In a second experiment, the progress of the inactivation was followed versus time. Granule membranes were preincubated with bromophenacyl bromide under the conditions detailed above. Replicate samples were removed from this reaction mixture at various times and assayed, both under nonstimulated and under glycol-stimulated conditions, for PLA_2 activity remaining. Both sets of assay conditions showed that the inactivation reaction obeyed first-order kinetics. However, while a half-time for inactivation of 95 min was observed for samples assayed under nonstimulated conditions, samples from the same reaction mixture assayed under the glycol-stimulated condition exhibited a $t_{1/2}$ of 190 min. These results provided

Table I: Copurification of Glycol-Stimulated PLA_2 Activity with Membrane-Bound Dopamine β-Hydroxylase in the Standard Preparation of Chromaffin Granule Membranes[a]

fraction separated	separation step		resultant fractions	protein (mg)	marker sp act. (nmol h^{-1} μg^{-1}) G6Pase	AChE	MAO	β-gluc	membrane-bound DβH	PLA_2 (% conversion) nonstim	glycol-stim
	800*g*	a	pellet	203	0.32	1.4	0.022	0.08	2.2	6.4	0.5
		b	supernatant								
b	26000*g*	c	supernatant	276	0.08	0.8	0.004	0.02	0.6	6.2	0.4
		d	pellet	119	0.38	2.6	0.096	0.12	17	5.2	4.5
c	100000*g*	e	supernatant	216	0.01	0.6	(0)	0.01	0.2	4.4	0.06
		f	pellet	30	0.60	2.8	0.034	0.10	7.0	5.2	3.3
d	1.8 M sucrose	g	buoyant fraction	112	0.30	3.4	0.115	0.12	29	5.6	3.5
		h	pellet	4.1	0.12	2.0	0.024	0.04	36	2.7	7.4
h	lysis	i	soluble fraction	3.3					0.6		0.06
		j	membrane	1.4	0.32	2.9	0.074	0.01	71	2.9	8.5
j	washing	k	membrane	0.9	0.35	2.6	0.088	0.03	79	5.0	11.8

[a] Chromaffin granule membranes were prepared from bovine adrenal medullae by the standard procedure. Samples were collected at each stage of the fractionation and assayed for protein, for PLA_2 activities, and for glucose-6-phosphatase (G6Pase), acetylcholinesterase (AChE), monoamine oxidase (MAO), and β-glucuronidase (β-gluc). Membrane-bound dopamine β-hydroxylase (DβH) was measured in pellets prepared from the fractions after lysis, using a Beckman airfuge at 25 psi, 20 min. Nonstimulated PLA_2 activity was measured in 90-min incubations containing 0.1 mM [^{14}C]PC liposomes and 0.14 mg/mL protein plus 0.3 M sucrose, 0.25 mM EDTA, 2.5 mM $CaCl_2$, and 50 mM Tris-HCl, pH 9.0. Incubations for measurement of glycol-stimualted PLA_2 activity were for 60 min and contained 0.05 mM [^{14}C]PC liposomes and 2.0 μg/mL membrane protein plus 50% (v/v) glycerol, 0.25 mM EDTA, 1 mM $CaCl_2$, 50 mM Tris-HCl, pH 9.0, and 0.03 mg/mL bovine serum albumin. All the values in the table represent averages of duplicate determinations and are given as specific activities relative to the total (soluble plus membrane-bound) protein in the fraction.

strong evidence that the membrane preparation contained more than one PLA_2 enzyme, differing in their rates of reaction with bromophenacyl bromide. Further, they suggested that the assay conducted under nonstimulated conditions did not accurately reflect the activity of the glycol-stimulable PLA_2.

Copurification of Glycol-Stimulated PLA_2 Activity with Chromaffin Granule Membranes. Studies regarding the subcellular compartmentation of neutral or alkaline-active PLA_2 in chromaffin tissue have not been reported to date. In various other mammalian cells, though, membrane-bound forms of the enzyme have been found in virtually every compartment including mitochondria, plasma membrane, Golgi, endoplasmic reticulum, lysosomes, and nuclei (Brockerhoff & Jensen, 1974; van den Bosch, 1980; Tamiya-Koizumi et al., 1989). It was therefore important to determine whether the PLA_2 activities detected in the granule membrane fraction were derived from the secretory granules themselves or from any other organelles possibly contaminating the preparation.

Analysis of marker enzymes in the subcellular fractions of chromaffin tissue demonstrated that, although the chromaffin granule fraction (1.8 M sucrose pellet) was highly enriched in dopamine β-hydroxylase, there was also a moderate degree of contamination of the fraction by other organelles (Table I). Lysis of the granules permitted removal of the soluble proteins, which constituted 70–80% of the protein in the granule fraction. Accordingly, the specific activities of the membrane-bound marker enzymes were increased in the initial and the final extensively washed granule membrane pellets compared to the intact granules. These markers indicated that this final preparation of granule membranes also contained residual amounts of mitochondrial, endoplasmic reticular, lysosomal, and plasma membranes.

The distributions of the marker enzymes in this fractionation were compared to the distribution of PLA_2 activity, measured under both nonstimulated and glycol-stimulated conditions. Because of the aforementioned nonlinearity of PLA_2 activity with respect to protein concentration (Figure 1), care was taken to assay an equal amount of protein in every sample. A significant portion of the nonstimulated PLA_2 activity appeared in the cytosol fraction (100000*g* supernatant), whereas this fraction contained negligible amounts of the glycol-stimulated enzyme. Both of these activities were found in the 26000*g* and 100000*g* pellet fractions. In the 1.8 M sucrose cushion step of the procedure, the glycol-stimulated PLA_2 was more highly enriched in the pellet fraction than in the buoyant fraction, whereas for nonstimulated PLA_2 activity the converse was true. This step also enriched the pellet, relative to the buoyant fraction, for the secretory granule markers, dopamine hydroxylase, and catecholamines. All the other marker enzymes showed depletion in the pellet compared to the buoyant fraction. It can be similarly seen that glycol-stimulated PLA_2 activity together with the granule markers was more highly enriched in the 1.8 M sucrose pellet than in the 100000*g* pellet, while this was not the case for any of the other marker enzymes or for the nonstimulated PLA_2 activity. On the basis of these findings, the glycol-stimulated PLA_2 activity appeared to copurify with the chromaffin granules. However, the nonstimulated PLA_2 activity had a different distribution that was not consistent with its being situated primarily in chromaffin granules.

Whereas the nonstimulated activity was observed in both soluble and particulate fractions, glycol-stimulated PLA_2 appeared to be exclusively membrane-bound. Upon disruption of the chromaffin granules by hypotonic lysis and freeze/thaw, the supernatant fraction representing the soluble contents of the secretory granules contained negligible glycol-stimulated PLA_2.

To verify the above indications that glycol-stimulated PLA_2 activity copurified with chromaffin granule membranes, the dense organelle fraction (26000*g* pellet) was applied to a sucrose density step gradient, and the distribution of PLA_2 activity was examined (Figure 3). Catecholamines released from ruptured granules remained in the sample overlay region after centrifugation. The amount indicated that approximately two-thirds of the chromaffin granules remained intact (data not shown). Catecholamines appearing at the top of the gradient in fraction 1 probably represented carryover from the overlay during sampling from the gradient. Membrane-bound components resolved on the gradient into four distinct peaks showing enrichment for markers of plasma membrane (fraction 1), mitochondria (fraction 4), lysosomes (fraction 7), or intact chromaffin granules (fraction 10). PLA_2 activity measured in the fractions under nonstimulated conditions showed a rather broad distribution, with the highest specific activity

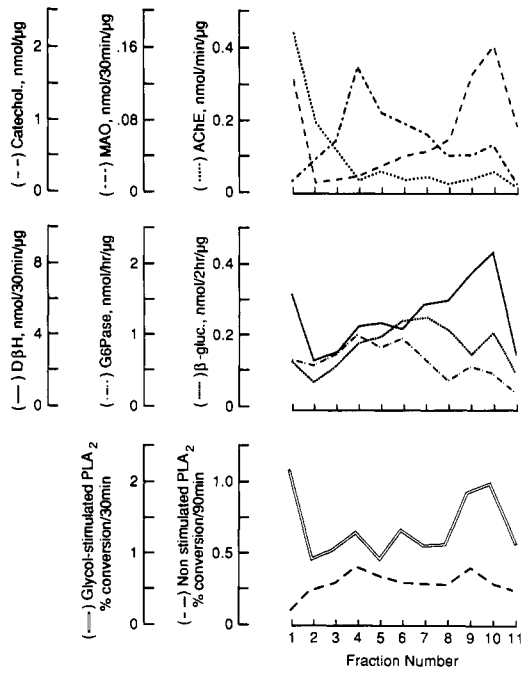

FIGURE 3: Distribution of glycol-stimulated PLA_2 activity on density gradients coincides with markers for the chromaffin granule. The 26000*g* pellet fraction from bovine adrenal medulla was resolved on sucrose step gradients (see Experimental Procedures). Samples were collected from the top (fraction 1) to the bottom (fraction 10) of the gradient; fraction 11 denotes the pellet at the bottom of the tube. Marker activities and PLA_2 activities measured in these fractions are plotted in three separate panels for clarity. Catecholamines, acetylcholinesterase, glucuronidase, monoamine oxidase, and glucose phosphatase were assayed in samples taken directly from the gradient fractions after freeze/thaw lysis. Membrane-bound dopamine β-hydroxylase and PLA_2 activities were measured in 40000*g* pellets prepared from each of the fractions after lysis, and using equal amounts of the membrane protein in each incubation. Nonstimulated PLA_2 was assayed in 90-min incubations containing 0.1 mM [^{14}C]PC liposomes, 0.15 mg/mL membrane protein, 0.25 M sucrose, 50 mM Tris-HCl, pH 9.0, 1 mM EDTA, and 5 mM $CaCl_2$. Incubations for glycol-stimulated PLA_2 were for 30 min and contained 0.04 mg/mL membrane protein, and 50% glycerol in place of sucrose. The values represent averages for duplicate determinations, and all are expressed as enzyme specific activities relative to the total (membrane-bound plus soluble) protein in the gradient fraction.

occurring in fraction 4. This suggested a mitochondrial location for at least a part of this activity. The specific activity of glycol-stimulated PLA_2, like that of membrane-bound dopamine hydroxylase, was highest in fraction 10, clearly demonstrating its presence in the chromaffin granule. This PLA_2 activity and that of dopamine hydroxylase were also enriched in fraction 1. This fraction, at the top of the gradient, would be the expected position for ghosts formed by lysis of chromaffin granules (Terland & Flatmark, 1980). However, this fraction was also highly enriched in plasma membranes, and in addition would be the expected position for Golgi membranes (Trifaro & Duerr, 1974). Thus, at this point, we cannot exclude the possibility that the same enzyme or a similar one may occur in the Golgi or in the plasma membrane, in addition to the membranes of the secretory granules.

A second criterion was used to confirm the copurification of the glycol-stimulated PLA_2 activity with granule membranes. Because these membranes are particularly low in protein content, they can be separated from the membranes of other organelles by flotation on 1.0 M sucrose (Schneider, 1972; Terland & Flatmark, 1980). This technique was used to further purify the granule membrane pellet obtained after osmotic lysis of the intact granules, and the distributions of glycol-stimulated PLA_2 and of marker enzymes in the resulting

Table II: Cofractionation of Glycol-Stimulated PLA_2 Activity with Dopamine β-Hydroxylase in the Purification of Chromaffin Granule Membrane Ghosts on 1.0 M Sucrose[a]

fraction	marker sp act. (nmol h^{-1} μg^{-1}) MAO	β-gluc	DβH	glycol-stim PLA_2 (% conversion)
initial suspension	0.086	0.018	126	4.5
sample overlay	(0)	0.001	14	0.94
interface	0.048	0.011	142	8.6
pellet	0.072	0.025	63	3.8

[a] Chromaffin granules prepared according to the standard procedure were lysed by freeze/thaw in hypotonic buffer (15 mM Tris-HCl, pH 7.4). The membrane ghosts were pelleted at 40000*g*, 45 min and then resuspended at 1.5 mg of protein/mL in the hypotonic buffer. The membrane suspension (1 mL) was laid over a cushion (6 mL) of 1.0 M sucrose/15 mM Tris-HCl, pH 7.4, and centrifuged at 80000*g*, 90 min. Fractions removed by pipet were assayed for monoamine oxidase (MAO), glucuronidase (β-gluc), and dopamine β-hydroxylase (DβH). Glycol-stimulated PLA_2 activity in the fractions was determined under conditions described in Table I. The specific activities in the table represent averages of duplicate determinations.

fractions are given in Table II. The 1.0 M sucrose interface fraction, containing 37% of the protein, was found to be enriched in both glycol-stimulated PLA_2 and dopamine β-hydroxylase, compared to the initial membrane suspension. In contrast, the specific activities of monoamine oxidase and of β-glucuronidase were reduced in the interface fraction but enriched in the denser membrane pellet. Again, then, the distribution of the glycol-stimulated PLA_2 coincided with that of the marker for granule membranes, dopamine β-hydroxylase, but not with markers for mitochondria or lysosomes.

Properties of the Glycol-Stimulated PLA_2. With some 30-fold stimulation of PLA_2 activity by addition of 50% glycerol, the sensitivity of the assay was enhanced to the extent that activity could be readily measured by using 1 µg/mL of granule membrane protein, or less. It also followed that under glycol-stimulated conditions, no more than 3% of the product was formed by nonstimulable forms of PLA_2. Thus, these reaction conditions provided an essentially selective assay for the PLA_2 enzyme that was found to be a component of the granule membrane. It was, therefore, possible to assess certain properties of the glycol-stimulated PLA_2 directly in the impure preparation of chromaffin granule membranes.

When dilute membrane suspensions were assayed in the presence of 50% glycerol, the stimulated PLA_2 activity proved to be linear vs time for at least 2 h, and was also linear with respect to granule membrane concentration over the range 0–2 µg of protein/mL (data not shown). A carrier protein (0.1 mg/mL bovine serum albumin) was routinely included in the buffer as a precaution against adsorptive losses in these very dilute suspensions of the membranes. The activity was largely unaffected by additions of KCl or NaCl to incubations at concentrations as high as 1.0 M (data not shown). The absence of interference by changes in ionic strength permitted determination of the pH dependence of the activity (Figure 4). The pH profile was symmetric, with optimal activity observed at pH 7.8. This result would be in keeping with an active-site histidine residue, as predicted by the susceptibility of the enzyme to bromophenacyl bromide inactivation.

Substrate specificity of the glycol-stimulated PLA_2 was also investigated. The acyl chain composition of the phosphatidylcholine used to prepare the carrier liposomes was found to influence the activity, and among various phospholipids tested, the best activity was obtained with diarachidonylphosphatidylcholine as the carrier phospholipid (data not shown). In Table III are shown the results obtained comparing different 2-acyl-labeled phospholipids as substrates, when each

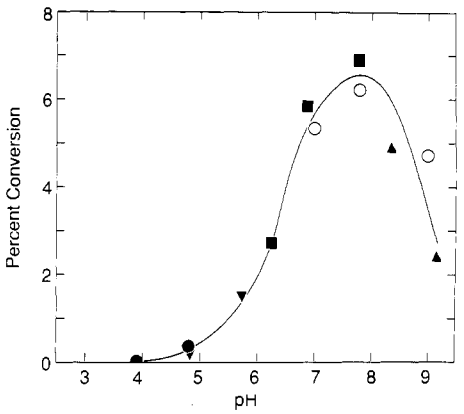

FIGURE 4: pH dependence of glycol-stimulated PLA_2. Incubations (60 min) were buffered with 0.1 M acetate (●), 4-morpholinoethanesulfonate (▼), *N*-(2-hydroxyethyl)-1-piperazineethanesulfonate (■), or glycine (▲) with sodium as the counterion, or with 0.1 M Tris (○) with chloride as the counterion. Other additions included 0.4 M NaCl, 50% glycerol, 0.25 mM EDTA, 1 mM $CaCl_2$, 1.9 μg/mL granule membranes, and 0.05 mM [^{14}C]PC liposomes. Each pH indicated was measured in the reaction mixture with an electrode at room temperature. Nonenzymatic product formation was less than 0.05% conversion over the entire pH range tested. The values shown represent averages for duplicate determinations.

Table III: 2-Arachidonylphosphatidylcholine Is a Preferred Substrate for Glycol-Stimulated PLA_2[a]

labeled compd	PLA_2 act. (% conversion)	
	minus glycerol	plus glycerol
1-16:0,2-[^{14}C]-20:4-PC	0.3	13.9
1-16:0-2-[^{14}C]-18:1-PC	0.3	4.0
di-[^{14}C]-18:0-PC	0.1	0.1
1-acyl-2-[^{14}C]-20:4-PE	0.3	4.2

[a] Liposomes of diarachidonylphosphatidylcholine containing 5 mol % of the labeled compound were tested individually as substrates in incubations (60 min) containing 0.1 mM liposomes and 2 μg/mL granule membrane protein plus 0.25 mM EDTA, 1 mM $CaCl_2$, 100 mM Tris-HCl, pH 8.0, and either 0 or 50% (v/v) glycerol. The values are averages of duplicate determinations, expressed as the net percent conversion (mol/mol) of the labeled compound to free fatty acid, after subtraction of nonenzymatic blanks.

was carried in diarachidonyl-PC liposomes. Under the conditions optimized for measurement of glycol-stimulated PLA_2, the activity in the granule membrane preparation in the absence of glycerol was negligible toward any of the labeled phospholipids. In the presence of glycerol stimulation, the highest PLA_2 activity was observed toward phosphatidylcholine bearing arachidonic acid in the 2-position. 2-Arachidonylphosphatidylethanolamine and 2-oleylphosphatidylcholine were also recognized as substrates, but exhibited lower activity. Negligible activity was obtained with phosphatidylcholine containing labeled stearoyl moieties. These results suggested that the glycol-stimulated enzyme requires an unsaturated acyl residue in the 2-position, and is especially active toward 2-arachidonyl species. Both the phosphocholine and phosphoethanolamine head groups were acceptable, although the substrate containing the latter moiety gave somewhat lower activity.

Discussion

In the work presented here, we have demonstrated the presence of PLA_2 enzymes in subcellular fractions of chromaffin tissue that may play a role in exocytosis. With the assay conditions and substrate selected, chromaffin granule membranes were shown to contain tightly membrane-bound PLA_2 that catalyzes the release of arachidonic acid. Alternative lipolytic pathways leading to formation of this metabolite were not detected. This activity was stimulated severalfold by addition of Ca^{2+}. The half-maximal Ca^{2+} concentration for this stimulation, 0.1–0.2 mM, was similar to that measured for the Ca^{2+}-dependent mixing of phospholipids between the bilayers of the synthetic PC liposomes and the chromaffin granule membranes (Hildebrandt and Albanesi, unpublished results). We do not know whether or not this liposome/membrane fusion is a requisite step prior to enzymatic hydrolysis in this heterogeneous assay. Thus, it is not certain whether the observed stimulation of activity by Ca^{2+} is actually due to the presence of a Ca^{2+}-dependent PLA_2 in chromaffin tissue, or whether it is an artifact of the assay. Because of the critical role of Ca^{2+} in triggering the exocytotic response in chromaffin cells, the unambiguous demonstration of Ca^{2+}-dependent PLA_2 activity in this tissue will be a relevant finding.

We have also shown that PLA_2 activity in the granule membrane fraction is very strongly stimulated by the presence of glycols, including glycerol, ethylene glycol, and poly(ethylene glycol). A similar, but more modest, stimulatory effect of glycerol on the activity of the solubilized PLA_2 from macrophage membranes has been noted (Ulevitch et al., 1988). The enhancement of activity in our preparation did not result from enzyme stabilization by the compounds, because the activity was reasonably stable in the absence of any glycol addition. Although the mechanism of this stimulatory effect of glycols is presently unknown, it has proven to be a valuable tool to distinguish a membrane-bound form of PLA_2 that is clearly compartmentalized to the secretory granules. Occurrence of the glycol-stimulated PLA_2 in Golgi or plasma membrane cannot yet be ruled out.

A significant amount of PLA_2 activity was measurable in the preparation under conventional assay conditions (Table I). Although this non-glycol-stimulated activity copurified with chromaffin granule membranes, in agreement with a previous report (Husebye & Flatmark, 1987), we did not find this fraction to be the most highly enriched. Significant activity could be detected in virtually every fraction, both soluble and particulate. The distribution of PLA_2 activity on sucrose density gradients indicated the occurrence of mitochondrial and lysosomal forms of the enzyme, as have also been found in other tissues (Brockerhoff & Jensen, 1974; van den Bosch, 1980). The chromaffin granule membrane pellet showed measurable cross-contamination by membranes derived from these organelles, and it is distinctly possible that these account for some or all of the activity found in the preparation. A portion of the PLA_2 activity in this membrane fraction was inhibited by rather low concentrations of salt, and in this respect resembled the pH 6.5 optimal PLA_2 described by Bartolf and Franson (1984), although the latter was reported to be a soluble enzyme. Due to the nonlinearity of activity with respect to protein concentration, we cannot calculate the contribution of enzyme units from contaminating lysosomes or other organelles.

Because the glycol-stimulated PLA_2 could be selectively assayed, we were able to characterize it directly in the granule membrane preparation. The enzyme shared the susceptibility to inactivation by bromophenacyl bromide which typifies many mammalian PLA_2 enzymes and which indicates an active-site histidine residue. The pH optimum for in vitro activity was about 7.8, and significant activity would be predicted at physiological pH. In this respect, the glycol-stimulated PLA_2 appears to differ from the granule membrane PLA_2 described by Husebye and Flatmark (1987); the latter showed highest

activity above pH 8.5. The glycol-stimulated PLA_2 was clearly active in the absence of free calcium, and so Ca^{2+} does not appear to be involved in the catalytic mechanism. Nevertheless, Ca^{2+} was seen to influence the activity, and may indeed play a regulatory role.

Three observations from the present study pertain to the potential involvement of the glycol-stimulated PLA_2 in the process of secretion. First, the unique location of the enzyme in the membranes of the secretory vesicles is apropos to generation of local high concentrations of fusogenic products—fatty acids and lysophospholipid—that may facilitate the fusion of the granule membrane with the plasmalemma. Second, the sensitivity of the enzyme activity toward glycols, agents which perturb the fluidity, surface potential, and other physical properties of phospholipid bilayers (Boni & Hui, 1987), suggests the responsiveness of the enzyme to its microenvironment. Third, the enzyme appears to be selective for phospholipids containing arachidonate in the *sn*-2 position, consistent with a proposed role in cellular signalling whereby PLA_2 is responsible for availability of the precursor in the biosynthesis of prostaglandins, leukotrienes, and/or hydroxy fatty acids.

In order to understand the function of the PLA_2 in the chromaffin granule membranes, it will be necessary to understand how the enzyme is regulated. Allosteric mechanisms, enzyme modification, and regulation through substrate availability are all possible means of controlling activity which remain to be explored. The knowledge acquired about the secretory process in chromaffin cells is expected to be applicable to other exocytotic systems, including insulin secretion, mast cell degranulation, and neurosecretion.

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Registry No. PLA_2, 9001-84-7; glycerol, 56-81-5; ethylene glycol, 107-21-1; poly(ethylene glycol), 25322-68-3; *p*-bromophenacyl bromide, 99-73-0; sucrose, 57-50-1; calcium, 7440-70-2; 1-palmitoyl-2-arachidonyl-PC, 35418-58-7; 1-palmitoyl-2-oleoyl-PC, 26853-31-6.

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Reciprocal Communication between the Lyase and Synthase Active Sites of the Tryptophan Synthase Bienzyme Complex†

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ABSTRACT: It is important to understand how the cleavage of indoleglycerol phosphate, which is catalyzed by the α subunits in the $\alpha_2\beta_2$ bienzyme complex of tryptophan synthase, is modulated by the presence of L-serine in the β subunits. Steady-state kinetic data, including the dependence of k_{cat} on pH, allowed values to be assigned to each of the eight rate constants of the minimal catalytic mechanism. An ionizing group having an apparent pK value near 7.5 must be protonated for activity. The α active site ligands indolepropanol phosphate, glyceraldehyde 3-phosphate, and glycerol 3-phosphate increase both the affinity and the molar absorbance of L-serine and L-tryptophan bound to the β active site. These effects prove that the α sites communicate with the β sites over a distance of 30 Å. 6-Nitroindole readily condenses with glyceraldehyde 3-phosphate, but not with L-serine. The turnover numbers for 6-nitroindoleglycerol phosphate and 6-nitroindole increased about 10-fold in both directions in the presence of L-serine bound to the β_2 subunits. These data prove that the α and β active sites communicate reciprocally and explain why the turnover number for the physiological reaction of indoleglycerol phosphate with L-serine greatly exceeds that of the cleavage reaction of indoleglycerol phosphate.

Tryptophan synthase from *Escherichia coli* (EC 4.2.1.20) is a simple bienzyme complex that catalyzes the final two steps in the biosynthesis of L-tryptophan. The enzyme is an $\alpha_2\beta_2$ tetramer, which can be dissociated easily into monomeric α subunits and the dimeric β_2 subunit. The latter contains one molecule of pyridoxal 5′-phosphate (PLP)[1] in each active site (Crawford & Yanofsky, 1958; Miles, 1991). The α subunit catalyzes the reversible cleavage of indoleglycerol phosphate (IGP) to indole (IND) and glyceraldehyde 3-phosphate (GAP); this is the "A" or "lyase" reaction:

$$\text{IGP} \rightleftarrows \text{IND} + \text{GAP} \qquad \text{(A)}$$

The β_2 subunit catalyzes the practically irreversible condensation of IND with L-serine to give L-tryptophan. This "B" or "synthase" reaction requires PLP:

$$\text{IND} + \text{Ser} \rightarrow \text{Trp} + \text{H}_2\text{O} \qquad \text{(B)}$$

The intact $\alpha_2\beta_2$ complex catalyzes the physiological AB reaction, which is formally the sum of the A and B reactions:

$$\text{IGP} + \text{Ser} \rightarrow \text{Trp} + \text{GAP} + \text{H}_2\text{O} \qquad \text{(AB)}$$

The isolated α and β_2 subunits are poor catalysts of their respective reactions. However, in the $\alpha_2\beta_2$ complex, the subunits become about 100-fold more active. This activation is contingent on conformational changes that occur during the assembly of the mature $\alpha_2\beta_2$ complex (Lane et al., 1984). The three-dimensional structure of tryptophan synthase shows that the α and β active sites are separated by about 30 Å (Hyde et al., 1988), confirming earlier indirect measurements (Heilmann & Holzer, 1981; Lane & Kirschner, 1983c). The

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; IGP, 3-indole-D-glycerol 3′-phosphate; IND, indole; GAP, D-glyceraldehyde 3-phosphate; Ser, L-serine; Trp, L-tryptophan; GP, DL-α-glycerol 3-phosphate; 6-nitro-IGP, (6-nitroindol-3-yl)-D-glycerol 3′-phosphate; 6-nitro-IND, 6-nitroindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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