Effects of Substitution of the Absolutely Invariant Glutamine-4 and Phenylalanine-5 in Bovine Pancreatic Phospholipase A_2 on Enzymatic Activity and Substrate Binding Properties†

Gustaaf J. M. van Scharrenburg, Wouter C. Puijk, Maarten R. Egmond, Peter H. van der Schaft, Gerard H. de Haas, and Arend J. Slotboom*,‡

ABSTRACT: The absolutely invariant character of Gln^4 and Phe^5 in all known phospholipase A_2 sequences strongly suggests that these residues fulfill important structural functions to stabilize the correct catalytic and/or substrate binding sites required for optimal activity of the enzyme. Although Met^8 is not absolutely invariant, its hydrophobic character is maintained in all phospholipases A_2. Semisynthesis was used to prepare three bovine pancreatic ϵ-amidinated phospholipase A_2 (AMPA) analogues in which Gln^4, Phe^5, and Met^8 were substituted by Nle^4, Tyr^5, and Nle^8, respectively. [Nle^8]-AMPA possesses almost identical properties as "native" AMPA, including enzymatic activity. However, substitution of Phe^5 by Tyr and Gln^4 by Nle in bovine phospholipase A_2 resulted in the almost complete loss of its catalytic activity when assayed on *micellar* L-dioctanoyllecithin. [Nle^4]AMPA still possesses about 25% of the catalytic activity of "native" AMPA toward *monomeric* bis(thiohexanoyl)lecithin, whereas hardly any activity (<4%) was detected for [Tyr^5]AMPA by using this assay. Direct binding studies using ultraviolet difference spectroscopy showed that [Tyr^5]AMPA in the presence of Ca^{2+} possesses an identical affinity for micelles of *cis*-9-octadecenylphosphocholine, as "native" AMPA, while no measurable affinity for these micelles could be detected for [Nle^4]AMPA. These findings strongly suggest that the absence of catalytic activity of [Tyr^5]- and [Nle^4]AMPA analogues is due to a distortion of the catalytic site of [Tyr^5]-AMPA, while for [Nle^4]AMPA it is due to the absence of the binding site for micelles. The results obtained from fluorescence spectroscopy, photo-CIDNP, ^1H NMR spectroscopy and immunological studies support the conclusion that the lipid binding site for micelles of [Tyr^5]AMPA is identical with that of "native" AMPA, whereas that of [Nle^4]AMPA is distorted.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of the 2-acyl ester linkage in 3-*sn*-phosphoglycerides (van Deenen & de Haas, 1964). The enzyme occurs widely spread in nature, most abundantly in snake venoms, and in mammalian pancreas (Verheij et al., 1981). In this latter source the enzyme is secreted as a zymogen, prophospholipase A_2, which upon limited proteolysis is converted to the active enzyme with the liberation of the N-terminal heptapeptide (Figure 1). Most of our present knowledge of various properties of the enzyme has been obtained from phospholipases A_2 purified from the above-mentioned two sources. At present about 30 complete amino acid sequences of phospholipases A_2 are known (Verheij et al., 1981), and from two of them the X-ray structures have been reported, viz., one of bovine pancreatic phospholipase A_2 (Dijkstra et al., 1978, 1981) and one of *Crotalus atrox* (Keith et al., 1981). On the basis of the 1.7-Å X-ray structure of bovine phospholipase A_2 and chemical modification studies, a mechanism for the catalysis was proposed (Verheij et al., 1980). Despite the knowledge of the mechanism of action, no satisfactory explanation is yet available for one of the most intriguing properties of phospholipase A_2, viz., its high catalytic activity toward substrate present as aggregated lipid–water interfaces. On the basis of the results of extensive investigations on pancreatic phospholipase A_2, it has been proposed (Verger et al., 1973; Pieterson et al., 1974a) that the enzyme undergoes a conformational change upon binding to the lipid–water interface, resulting in an enzyme form having more efficient catalytic properties. This hypothesis supposes a special site to be present in the enzyme involved in the binding to lipid–water interfaces: the lipid binding site or IRS.[1] The zymogen which does not bind to lipid–water interfaces lacks this site and possesses only low inherent catalytic activity toward monomeric substrate comparable to that of phospholipase A_2 (Pieterson et al., 1974a). Subsequent studies in our laboratory have shown that in addition to the N-terminal region of the porcine phospholipase A_2 Ala^1–Arg^6, Tyr^{69}, Leu^{19} (=Tyr^{19} in the horse phospholipase A_2), and Met^{20} are part of the IRS (van Dam-Mieras et al., 1975; Meijer et al., 1979; Meijer, 1979). In the N-terminal region Gln^4 and Phe^5 are absolutely invariant in all known phospholipase A_2 sequences (Figure 1), while the hydrophobic character of Met^8 is also conserved. Although these three amino acid residues are not involved in the catalytic mechanism, their highly invariant character suggests that they fulfill crucial functions in maintaining the

† From the Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, University Center "De Uithof", Padualaan 8, 3508 TB Utrecht, The Netherlands. *Received August 21, 1981.* These investigations were carried out under the auspices of The Netherlands Foundation for Chemical Research (SON) and with financial aid from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (1967, 1972) were used throughout. Other abbreviations: AMPA, ϵ-amidinated phospholipase A_2; AMPREC, ϵ-amidinated prophospholipase A_2; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; EDC, *N*-ethyl-*N'*-[3-(dimethylamino)-propyl]carbodiimide; HOBt, 1-hydroxybenzotriazole; iBuOCOCl, isobutyl chloroformate; F_3CCOOH, trifluoroacetic acid; DMF, dimethylformamide; For, formyl; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; LC, liquid chromatography; IRS, interface recognition site; CPK model, Corey–Pauling–Koltun space-filling molecular model; CIDNP, chemically induced dynamic nuclear polarization; TSP, [2,2,3,3-$^2H_4$]trimethylsilylpropionate; NMR, nuclear magnetic resonance; pH*, uncorrected pH meter readings in ^2H_2O; Pth, phenylthiohydantoin; RIA, radioimmunoassay; dpm, disintegrations per minute; cpm, counts per minute; ppm, parts per million.

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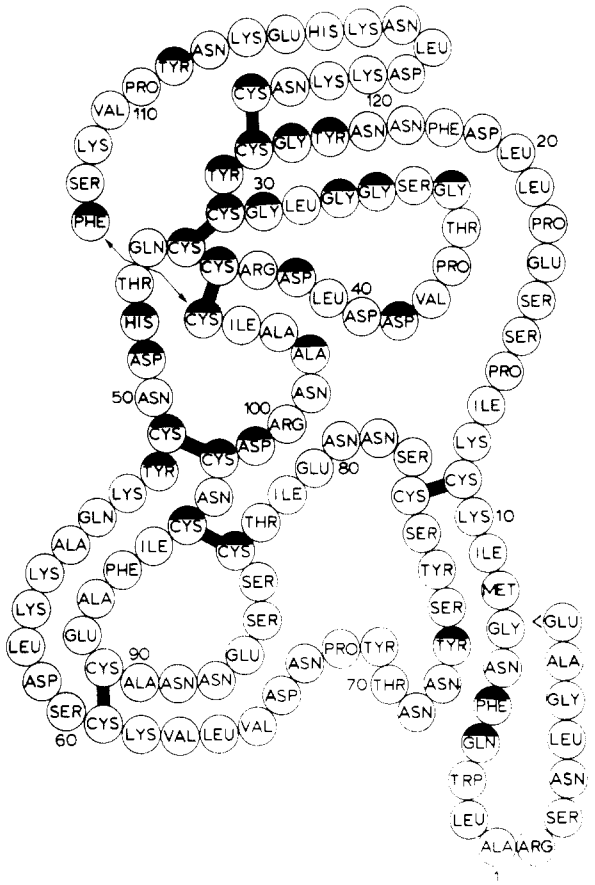

FIGURE 1: Primary structure of bovine pancreatic prophospholipase A_2 (residues in black are absolutely invariant in all phospholipases A_2) (Fleer et al., 1978).

correct conformation of the lipid binding site and/or the active site. This paper describes the influence of the substitution of Gln^4, Phe^5, and Met^8 by Nle, Tyr, and Nle, respectively, in bovine pancreatic phospholipase A_2 on its catalytic activity and substrate binding properties.

Experimental Procedures

Materials

Methylacetimidate hydrochloride was prepared according to the procedure of Hunter & Ludwig (1962). DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman (England), and various Sephadex products, AH-Sepharose 4B and CH-Sepharose 4B, were from Pharmacia Fine Chemicals (Sweden) and prepared for use according to the manufacturer's recommendations. Bovine pancreatic prophospholipase A_2 was purified from bovine pancreas (Dutilh et al., 1975). *N-t*-Boc derivatives of Gly, L-Asn, L-Ala, L-Leu·H_2O, L-Phe, and L-Gln were obtained from Vega Biochemicals (Tucson, AR). N^{α}-*t*-Boc-N^{in}-formyl-L-Trp and N^{α}-*t*-Boc-*O*-2-bromobenzyloxycarbonyl-L-Tyr were purchased from the Protein Research Foundation (Osaka, Japan), and N^{α}-*t*-Boc-L-Met-*N*-hydroxysuccinimide ester was from Fluka A.G. (Switzerland). N^{α}-*t*-Boc-L-Nle was prepared from L-Nle according to the procedure described by Moroder et al. (1976). N^{α}-*t*-Boc-L-Nle-*N*-hydroxysuccinimide ester was prepared from N^{α}-*t*-Boc-L-Nle by reaction with DCC and HOBt (Anderson et al., 1964). The purity of the various amino acid derivatives was checked by established procedures (thin-layer chromatography on silicic acid and melting points). Isobutyl chloroformate, *N*,*N*′-dicyclohexylcarbodiimide, *N*,*N*′-diisopropylethylamine, and 1-hydroxybenzotriazole were from Aldrich (Belgium). Dimethylformamide (Uvasol), methylene dichloride (pro analysi), Chloramine T (trihydrate), and *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide (zur Synthese) were products of Merck (Germany). Liquid HF was from Matheson, acetonitrile (high-performance LC quality) from Baker Chemicals, trifluoroacetic acid from Pierce, immunobead goat anti-rabbit immunoglobulin from Bio-Rad Laboratories, and ^{125}I from the Radiochemical Centre (Amersham, England). Trypsin and TPCK-treated trypsin were products from Serva (Germany). 1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine was prepared as described by Cubero Robles & van den Berg (1969). *cis*-9-Octadecenylphosphocholine and [2,3-bis(hexanoylthio)propyl]phosphocholine were synthesized as described by van Dam-Mieras et al. (1975) and Volwerk et al. (1979), respectively. 1-Bromo[2-^{14}C]octan-2-one was synthesized as described previously (Slotboom et al., 1978). ^{125}I-Labeled bovine AMPA was obtained from AMPA by reaction with ^{125}I using the Chloramine T procedure (McConahey & Dixon, 1980).

N-t-Boc-glycine-resin was prepared by esterification of *N-t*-Boc-glycine to chloromethylated polystyrene resin 1% cross-linked with divinylbenzene (S-XI, Bio-Rad; 1.34 mM Cl/g) according to the method of Gisin (1973).

Methods

Protein concentrations were calculated from the absorbance at 280 nm with an E_{1cm} (1%) of 12.3 for the bovine (pro)-phospholipase A_2, their ϵ-amidinated derivatives, and for the semisynthetic bovine [Nle^4]- and [Nle^8]AMPA's. The protein concentrations of semisynthetic bovine [Tyr^5]AMPA and of AMPA-specific γ-globulins were determined at 280 nm by using an E_{1cm} (1%) of 13.0 and 13.5, respectively.

Amino acid analyses were performed by the method of Spackman et al. (1958) on a Kontron Liquimat II amino acid analyzer equipped with a Kontron computing integrator. Samples were hydrolyzed in vacuum-sealed ampules with 5.8 N HCl for 24 h at 110 °C. The homogeneity of the proteins and protein fragments was checked by polyacrylamide gel electrophoresis at pH 9.6 as described before (van Scharrenburg et al., 1981).

N-Terminal amino acid residues of the protein fragments were determined by dansylation according to the method of Gray (1972). Automated sequence analysis of the semisynthetic AMPA analogues was performed with a Beckman sequencer, Model 890 C, using a slightly modified Quadrol program no. 122974. The thiazolinone derivatives of the amino acid residues were collected in tubes containing 0.2 mL of 0.1 M HCl and 0.1% ethyl mercaptan (v/v). The identification of the phenylthiohydantoin derivatives of the amino acid residues was done by high-performance LC as described by Frank & Strubert (1973). The enzymatic activities of bovine AMPA and its semisynthetic analogues were routinely determined by using the titrimetric assay procedure with egg yolk lecithin as substrate (Nieuwenhuizen et al., 1974). In contrast to the procedure, 2-fold higher Ca^{2+} and 1.5-fold higher sodium deoxycholate concentrations were used. Kinetic measurements using micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine and monomeric [2,3-bis(hexanoylthio)-propyl]phosphocholine were performed as described before (de Haas et al., 1971, and Volwerk et al., 1979, respectively).

The second-order rate constants (k_2) for the modification of the active-site histidine-48 of AMPA and [Tyr^5]AMPA were determined at pH 6.0 and 35 °C by using 1-bromo[2-^{14}C]octan-2-one (252 dpm/nmol) as described by Volwerk et al. (1974) and Volwerk (1979). Direct binding studies were carried out by using ultraviolet difference spectroscopy as previously described by Hille et al. (1981). The ultraviolet

difference spectra were recorded at 25 °C on an Aminco DW-2a spectrophotometer with an automatic base-line correction accessory (Midan data analyzer, from Aminco). $K_{Ca^{2+}}$ values were determined by using ultraviolet difference spectroscopy as described by Pieterson et al. (1974b). Fluorescence spectroscopy was done as described by van Dam-Mieras et al. (1975).

NMR Spectroscopy. The ^1H NMR (360 MHz) photo-CIDNP spectra obtained with a Bruker HX-360 spectrometer at the SON Facility in Groningen, The Netherlands, were recorded as described before (Kaptein, 1978; Jansen et al., 1978; Egmond et al., 1980) by using 3-(carboxymethyl)-lumiflavin as the dye. Chemical shifts were calculated relative to TSP by using 3.764 ppm for the chemical shift difference between TSP and dioxane. The latter was added as internal standard.

Preparation of Immunoadsorbent Columns of Bovine AMPA and Des(Ala^1–Gly^7)AMPA. AMPREC was quantitatively coupled via its carboxylates to AH-Sepharose 4B. Therefore, 2 g of AH-Sepharose 4B was swollen in 10 mL of H_2O (pH 4.5), and subsequently 170 mg of AMPREC and 40 mg of EDC were added. This reaction mixture was incubated for 24 h at 4 °C. After being coupled, the bound AMPREC was converted into AMPA by limited tryptic proteolysis (de Haas et al., 1968). In contrast to AMPREC, des(Ala^1–Gly^7)AMPA was coupled via its α-NH_2 group to CH-Sepharose 4B. Coupling was performed in an 0.1 M $NaHCO_3$–buffer, pH 8.0, as described above, and 75% of the des(Ala^1–Gly^7)AMPA was found to be attached to the CH-Sepharose 4B.

Isolation of N-Terminal-Directed Bovine AMPA Specific γ-Globulins. Antibodies against bovine ϵ-amidinated phospholipase A_2 were raised by injecting New Zealand white rabbits (2 kg) subcutaneously in the back with an initial dose of 1 mg of protein emulsified in complete Freund's adjuvant.

The level of antibody titer was determined 1 week after each booster injection with a quantitative microcomplement fixation assay (Levine & van Vunakis, 1967) or with a hemagglutination assay (Overbeeke et al., 1980). After about 4 months the maximal level of antibody titer was achieved, and the blood was collected by heart puncture. The complement of the antisera was inactivated by heating for 30 min at 56 °C.

Bovine AMPA specific γ-globulins were isolated by ammonium sulfate precipitation and affinity chromatography using the column of bovine AMPA–AH-Sepharose 4B, as described previously for porcine phospholipase A_2 (Meijer et al., 1978). The isolation of the bovine AMPA specific γ-globulins directed to the N-terminal part of the protein molecule was done similarly by using the immunoadsorbent consisting of des(Ala^1–Gly^7)AMPA–CH-Sepharose 4B. To this column (0.8 × 4 cm) 50 mg of AMPA-specific γ-globulins was applied in a buffer of 20 mM Tris-HCl and 0.15 M NaCl, pH 7.4, at 4 °C.

The N-terminal-directed fraction of the γ-globulins, which was not bound to the column, eluted in the dead volume. The residual fraction of the γ-globulins bound to the immunoadsorbent column (about 65%) was eluted with 0.53 M formic acid and 0.15 M NaCl, pH 2.05, neutralized to pH 7.4 immediately after elution, and dialyzed against 20 mM Tris-HCl buffer, pH 7.4, and 0.15 M NaCl at 4 °C.

Radioimmunoassay. Bovine [^{125}I]AMPA (50 cpm/ng), the semisynthetic bovine AMPA's, bovine des(Ala^1–Gly^7)AMPA, and the N-terminal-directed γ-globulins were diluted with RIA buffer (20 mM Tris-HCl, pH 7.2, 0.15 M NaCl, and 0.1% BSA) to final concentrations of 1.2, 1.0, 1.0, and 10 μg of protein/mL, respectively. From these dilutions 30 μL of [^{125}I]AMPA solution and 400 μL of N-terminal-directed γ-globulin solution were mixed with increasing volumes (0–400 μL) of the diluted semisynthetic proteins. To these dilutions was added RIA buffer to a final volume of 1 mL, and the samples were subsequently incubated at 37 °C. After 1 h of incubation, 400 μL of second antibody (goat anti-rabbit immunoglobulin beads of Bio-Rad, diluted with RIA buffer, according to the manufacturer's instructions) was added to each sample. The samples were then incubated for 2 h at 37 °C, diluted with 1 mL of RIA buffer, and centrifuged for 10 min at 2500 rpm. The beads of each sample were separated from the solution, and their radioactivity was measured by counting for 20 min in a Packard Auto-γ spectrometer.

Preparation of Bovine AMPREC, AMPA, and Des(Ala^1–Met^8)AMPA. These ϵ-amidinated proteins and the protein fragment were prepared as described recently (van Scharrenburg et al., 1981).

Preparation of the ϵ-Amidinated Protein Fragments Met^8·Ile^9–Cys^{123} and Nle^8·Ile^9–Cys^{123}. These protein fragments were prepared by coupling bovine des(Ala^1–Met^8)-AMPA with the *N*-hydroxysuccinimide esters of *N-t*-Boc-L-Met and *N-t*-Boc-L-Nle, respectively (Slotboom & de Haas, 1975). After deblocking with trifluoroacetic acid and purification on CM-cellulose, both fragments were homogeneous on gel electrophoresis and had a correct amino acid composition. The dansyl procedure showed Met, respectively Nle, to be present as the only N-terminal amino acid residue in these fragments.

Synthesis of Peptides t-Boc-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly, t-Boc-Ala-Leu-Trp(For)-Gln-Tyr-Asn-Gly, and t-Boc-Ala-Leu-Trp(For)-Nle-Phe-Asn-Gly. The peptides were prepared by solid phase synthesis (Erickson & Merrifield, 1976) using a computerized peptide synthesizer (SYN 1) developed by the Danish Institute of Protein Chemistry (Villemoes et al., 1978). The peptide syntheses were done, starting with *N-t*-Boc-glycine-resin (1.17 mmol of *t*-Boc-Gly/g of polystyrene resin 1% cross-linked with divinylbenzene) using essentially the same automatic protocol for coupling and deprotection as described in detail by van Scharrenburg et al. (1981). During synthesis, the indole nitrogen of tryptophan was protected by a formyl group (Yamashiro & Li, 1973b) and the phenolic hydroxyl of tyrosine by an *O*-2-bromobenzyloxycarbonyl group (Yamashiro & Li, 1973a). After completion of the syntheses, amino acid analysis of the *N-t*-Boc-peptide-resins showed an average yield of 80%, as determined by the method of Westall et al. (1972). Peptides were cleaved from the solid support by treatment with liquid HF in the presence of 10% anisole using an HF-Reaction apparatus type I (Peptide Institute, Inc., Osaka, Japan). After removal of the HF in vacuo, the anisole was extracted with ethyl acetate (3 × 10 mL). Extraction of the peptides was done by successive treatments with 10 mL of acetic acid, acetic acid–H_2O [1:1 (v/v)], and water (4 times). The combined extracts were diluted with water and lyophilized. The overall yield after the extraction was about 75% for the peptides H_2N-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly and H_2N-Ala-Leu-Trp(For)-Gln-Tyr-Asn-Gly. The yield of the peptide H_2N-Ala-Leu-Trp(For)-Nle-Phe-Asn-Gly was only 20%.

Purification of Peptides. The purification of the peptides H_2N-Ala-Leu-Trp(For)-Nle-Phe-Asn-Gly and H_2N-Ala-Leu-Trp(For)-Gln-Tyr-Asn-Gly was done by LC on a C_{18} reversed-phase Polygosil column (50 × 2.5 cm, particle size 25–40 μm). The column was equilibrated with 10% aceto-

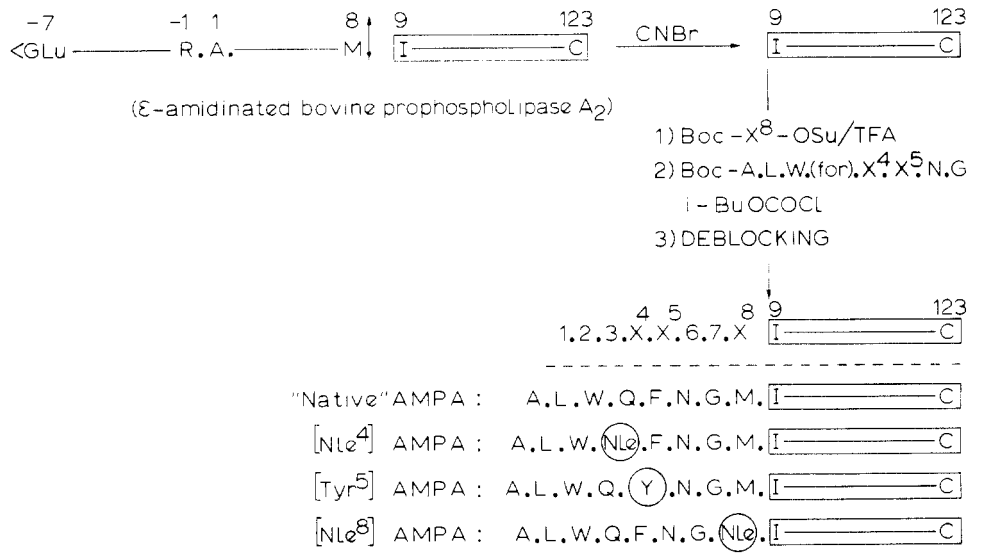

FIGURE 2: Scheme depicting semisynthesis of ε-amidinated bovine phospholipase A_2 (AMPA) analogues. "Native" AMPA (X^4 = Q; X^5 = F; X^8 = M); [Nle^4]AMPA (X^4 = Nle; X^5 = F; X^8 = M); [Tyr^5]AMPA (X^4 = Q; X^5 = Y; X^8 = M); [Nle^8]AMPA (X^4 = Q; X^5 = F; X^8 = Nle). Boc, OSu, for, TFA, and i-BuOCOCl refer to *tert*-butyloxycarbonyl, hydroxysuccinimide ester, formyl, trifluoroacetic acid, and isobutyl chloroformate, respectively.

nitrile in water containing 0.1% F_3CCOOH at room temperature; 50 mg of the peptides, dissolved in 10 mL of 10% acetonitrile in water containing 0.1% F_3CCOOH, was loaded on the column and eluted with a linear gradient from 10% to 90% acetonitrile in water containing 0.1% F_3CCOOH (2 × 250 mL). Fractions containing the purified peptides were pooled, diluted with distilled water, and lyophilized. The peptide H_2N-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly was purified on a column containing chromobeads type P (Technicon) at 55 °C and 560 psi (flow rate 1 mL/min) and by high-performance LC on C_{18} reversed phase as described before (van Scharrenburg et al., 1981). Subsequently, the α-amino group of the peptides was blocked with the *t*-Boc group by reacting the peptides with di-*tert*-butyl dicarbonate (Morodor et al., 1976).

The reaction mixtures containing the peptides *N-t*-Boc-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly and the one with Phe replaced by Tyr were applied to a C_{18} reversed-phase Polygosil column equilibrated with 10% acetonitrile in water containing 0.1% F_3CCOOH and eluted as described above. The *N-t*-Boc-peptide containing fractions were pooled, diluted with water, and lyophilized. The *N-t*-Boc-protected Nle^4 peptide could not be purified by LC on C_{18} reversed-phase Polygosil, due to its very hydrophobic character. Therefore, this peptide was isolated from the reaction mixture by precipitation with ice-cold H_2O. After centrifugation, the *N-t*-Boc-peptide was washed several times with ether and the residue dissolved in 80% acetonitrile in H_2O and lyophilized. The homogeneity of the peptides after each preparative purification step was checked by analytical high-performance LC on a C_{18} reversed-phase Lichrosorb column using a gradient reaching 90% acetonitrile in H_2O as described recently (van Scharrenburg et al., 1981). The pure *N-t*-Boc-peptides obtained in a yield of 50–70% based on the crude material after HF cleavage showed correct amino acid analyses.

Preparation of Semisynthetic Bovine [Nle^4]-, [Tyr^5]-, and [Nle^8]AMPA and of Native AMPA. The α-amino-protected peptides were covalently attached to the ε-amidinated protein fragments Met^8-Ile^9–Cys^{123} and Nle^8-Ile^9–Cys^{123} by using the mixed carbonic anhydride method (Meienhofer, 1979) (Figure 2). The coupling procedure and subsequent deprotection as well as the purification of the semisynthetic proteins were done as described in detail by van Scharrenburg et al. (1981). N^{α}-*t*-Boc-[Tyr^5]AMPA was prepared similarly, omitting the deprotection with trifluoroacetic acid.

The semisynthetic bovine [Nle^4]-, [Tyr^5]-, N^{α}-*t*-Boc-[Tyr^5]-, and [Nle^8]AMPA's and the native AMPA were obtained in about 30% yield and were homogeneous on gel electrophoresis at pH 9.6. The N-terminal sequence of these semisynthetic proteins as determined by automated Edman degradation was found to be in good agreement with the desired sequence.

Results and Discussion

Usually chemical modification experiments in nonmicrobial systems are limited to amino acid residues containing reactive side chains. Information about the importance of amino acid residues with inert side chains for the biological properties of a protein can only be obtained by X-ray analysis and NMR studies, or by a semisynthetic approach (Offord & DiBello, 1978; Tesser & Boon, 1980; Chaiken, 1981). Such a semisynthetic approach offers the possibility to substitute amino acid residues at the N-terminal region in bovine pancreatic phospholipase A_2 due to the presence of the unique Met residue at position 8. The residues Gln^4 and Phe^5 are absolutely invariant in all phospholipases A_2 sequenced so far. Met^8 is invariant in all pancreatic phospholipases A_2 while it is only occasionally replaced in some snake venom phospholipases A_2 by Leu or Val (Verheij et al., 1981). Although the residues Gln^4, Phe^5, and Met^8 are not directly involved in enzymatic hydrolysis, it seems very likely that they fulfill important structural functions, necessary for the enzyme to display its optimal activity. Therefore, the semisynthetic Nle^4-, Tyr^5-, and Nle^8-substituted ε-amidinated bovine phospholipase A_2 analogues were prepared by using the methods described recently by van Scharrenburg et al. (1981). Again ε-amidinated bovine phospholipase A_2 was used for these semisyntheses. This enzyme is almost identical with the native bovine phospholipase A_2 with respect to catalytic and binding properties. CNBr cleavage of ε-amidinated bovine prophospholipase A_2 yielded des(Ala^1–Met^8)AMPA, a protein devoid of all catalytic activity and which has only one free amino group, viz., the α-NH_2 group of Ile^9 (van Scharrenburg et al., 1981) (Figure 2). Covalent coupling of the *N*-hydroxysuccinimide esters of *N-t*-Boc-L-Met and *N-t*-Boc-L-Nle to des(Ala^1–Met^8)AMPA

Table I: Comparison of Various Properties of Semisynthetic Bovine ε-Amidinated Tyr[5]-, Nle[4]-, and Nle[8]-Substituted Phospholipases A_2 with Native and Semisynthetic ε-Amidinated Bovine Phospholipases A_2

	native AMPA	semisynthetic AMPA	[Tyr[5]]AMPA	[Nle[4]]AMPA	[Nle[8]]AMPA
sp act.[a] [μequiv min^{-1} (mg of protein)$^{-1}$] (egg yolk assay)	50 ± 3	46 ± 3	*f*	*f*	56 ± 3
V_{max}[b] [μequiv min^{-1} (mg of protein)$^{-1}$] (micellar L-dioctanoyllecithin)	2300 ± 160	2500 ± 50	60 ± 20	70 ± 20	2500 ± 50
k_{cat}/K_M[c] (s^{-1} M^{-1}) [monomeric bis(thiohexanoyl)lecithin]	172 ± 10	179 ± 10	5 ± 2	45 ± 5	228 ± 10
$K_{Ca^{2+}}$[d] (mM)	4.6 ± 0.1		5.2 ± 0.1	10 ± 1	2.7 ± 0.1
K_D[e] (mM) (binding of micellar *cis*-9-octadecenylphosphocholine)	5.0 ± 0.6	5.4 ± 0.6	5.0 ± 0.6	*g*	3.1 ± 0.4

[a] Determined at pH 8.0 as described under Methods. [b] Determined by titration of fatty acids liberated with 7.23 mM NaOH at pH 6.0 and 45 °C (50 mM $CaCl_2$, 10 mM acetate, and 0.1 M NaCl) as described previously (de Haas et al., 1971). Appropriate aliquots of enzyme solutions were added from stock solutions (0.2–0.4 mg of enzyme/mL) after a 50–100-fold dilution in distilled H_2O containing bovine serum albumin (10 mg/mL) to avoid irreversible adsorption to the glass. [c] Determined at pH 8.5 and 25 °C as described by Volwerk et al. (1979). [d] Determined by ultraviolet difference spectroscopy (Pieterson et al., 1974b). Conditions used: 50 mM acetate and 0.1 M NaCl, pH 6.0, 25 °C. [e] Determined by ultraviolet difference spectroscopy (Hille et al., 1981). Conditions used: 50 mM acetate, 0.1 M $CaCl_2$, and 0.1 M NaCl, pH 6.0, 25 °C (its concentration is expressed in lipid monomer concentration). [f] No detectable activity. [g] No detectable binding.

followed by deblocking with trifluoroacetic acid furnished the ε-amidinated fragments Met[8]·Ile[9]...Cys[123] and Nle[8]·Ile[9]...Cys[123], respectively (Slotboom & de Haas, 1975; van Scharrenburg et al., 1981). The N-terminal heptapeptides were restitched to the latter two protein fragments using the mixed carbonic anhydride method in order to obtain the desired AMPA analogues (Meienhofer, 1979) (Figure 2). Because Gly is the C-terminal amino acid in these heptapeptides, this coupling proceeds without any risk of racemization. After deprotection and purification, the semisynthetic bovine [Nle[4]]-, [Tyr[5]]-, and [Nle[8]]AMPA's as well as semisynthetic native AMPA were obtained in an average yield of about 30%. As can be seen from Table I the semisynthetic native enzyme is almost indistinguishable from the original bovine AMPA. The sequence analyses of the N-terminal part of the three analogues gave the expected results, including Trp (supplementary material) (see paragraph at end of paper regarding supplementary material). It can thus be concluded that the procedures applied are feasible and that the semisynthetic bovine [Nle[4]]-, [Tyr[5]]-, and [Nle[8]]AMPA's indeed contain the desired substitutions at the chosen loci.

According to the 1.7-Å X-ray structure of bovine phospholipase A_2 (Dijkstra et al., 1981), the residues Met[8] and Phe[5] are both part of the hydrophobic wall surrounding the catalytic site. Met[8] is located at the bottom of the catalytic site cavity behind Asp[99], which is thought to be directly involved in the catalytic action together with His[48] and the Ca^{2+}-binding ligand Asp[49] (Verheij et al., 1980). Substitution of this Met[8] residue (I) by a norleucine (II), thus replacing the sulfur atom

$$CH_3SCH_2CH_2CH(NH_3^+)COO^-$$
I

$$CH_3CH_2CH_2CH_2CH(NH_3^+)COO^-$$
II

by a methylene group, has almost no effect on the catalytic activity and binding properties of bovine AMPA as shown in Table I.

The immunological assay used gives information on conformational changes at that part of the surface region of the protein where the N-terminal region is located. As can be seen in Figure 3, no difference in response was found for [Nle[8]]-AMPA and native AMPA, indicating an identical conformation of the N-terminal region in both enzymes. Similarly, fluorescence spectroscopy of [Nle[8]]AMPA and native AMPA indicates a similar microenvironment for Trp[3] in both proteins (Figure 5). These results confirm previous findings (Meijer,

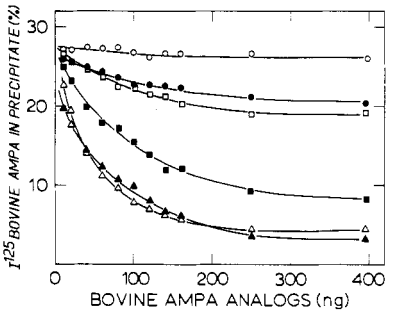

FIGURE 3: Comparison of the conformation of the N-terminal regions of bovine AMPA (△) and of (▲) [Nle[8]]-, (●) [Nle[4]]-, (■) [Tyr[5]]-, (□) α-*N*-*t*-Boc-[Tyr[5]]-, and (○) des(Ala[1]–Gly[7])AMPA's, using N-terminal-directed AMPA-specific γ-globulins in a radioimmunoassay (conditions as described under Methods).

1979; Dijkstra et al., 1981) that Met[8] is located in the interior of the protein. The fact that in some snake venom phospholipases A_2 Met[8] is replaced by a Leu or Val residue (Verheij et al., 1981) is in good agreement with the above-mentioned results and shows that the role of the sulfur atom in Met[8] is not critical for the functions of the enzyme.

In contrast to the Met[8] substitution, replacement of the absolutely invariant Phe[5] by a Tyr residue has a large effect on the catalytic properties of the enzyme (Table I). Substitution of Phe[5] by Tyr in bovine AMPA leads to an almost complete loss of enzymatic activity not only on micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine but also on monomeric [2,3-bis(hexanoylthio)propyl]phosphocholine. As can be seen also from Table I, the affinity of [Tyr[5]]AMPA for micelles of the substrate analogue *cis*-9-octadecenylphosphocholine is identical with that of native AMPA, indicating the presence of an intact interface recognition site in [Tyr[5]]AMPA. This finding is supported by the results obtained from photo-CIDNP ^{1}H NMR spectroscopy and from immunology. The photo-CIDNP method, which monitors exposed aromatic residues (Kaptein, 1978), reveals for bovine AMPA signals corresponding to Trp[3] and Tyr[69] (Jansen et al., 1978; Egmond et al., 1980) two amino acid residues, both belonging to the hydrophobic IRS (van Dam-Mieras et al., 1975; Meijer et al., 1979) (Figure 4, middle curve). For [Tyr[5]]AMPA a photo-CIDNP spectrum was obtained similar to that observed for native AMPA (Figure 4, lower curve). The RIA, using the N-terminal-directed AMPA-specific γ-globulins, can detect minor changes in antigenic properties. As can be seen from Figure 3, only a small decrease in the antigenic response is

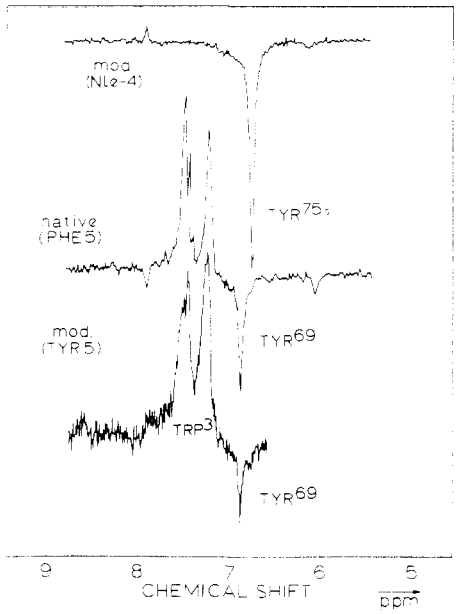

FIGURE 4: Photo-CIDNP ^{1}H NMR difference spectra of native (middle) and Nle^4- (upper) and Tyr^5-substituted (lower) ϵ-amidinated bovine phospholipases A_2. Conditions: 1.5–2 mM protein, 1.5 mM dioxane, and 0.2 mM flavin dye, 25 °C, pH* 5.0; ionic strength 0.28 M NaCl in D_2O. The small signal at 7.9 ppm (emission or absorption) originates from the flavin dye.

observed for [Tyr^5]AMPA as compared to native AMPA. A much larger decrease is found for [Tyr^5]AMPA in which the α-NH_2 group of Ala^1 is blocked by the *t*-Boc group. This observation is in good agreement with previous investigations where it was found that blocking of the α-NH_2 group of Ala^1 changes the conformation of the N-terminal region considerably. As a result of this modification, a functional IRS is no longer present, a situation very much resembling that of the zymogen (Slotboom & de Haas, 1975). Therefore, it can be concluded that [Tyr^5]AMPA has an intact IRS. The most plausible explanation for the absence of catalytic activity of [Tyr^5]AMPA must be a distortion of the active site. In this respect it has to be mentioned that the second-order rate constant for the incorporation of 1-bromo[2-^{14}C]octan-2-one on His^{48} of [Tyr^5]AMPA was found to be smaller by a factor of 5 as compared to that for native AMPA. It has been proposed that Phe^5, located near the entrance of the catalytic site cavity, partly shields the active-site couple His^{48}–Asp^{99} from the solvent (Dijkstra et al., 1981). The presence of the more polar tyrosine residue instead of Phe probably decreases this shielding effect, resulting in the loss of catalytic activity. In this respect it is of interest to note that almost all of the hydrophobic amino acid residues comprising the wall of the catalytic site are invariant in all phospholipases A_2. However, it cannot be excluded that a steric constraint of the active site due to the larger Tyr residue or that a direct interference of the aromatic hydroxyl group with the charge–relay system of the active-site couple His^{48}–Asp^{99} is responsible for the loss of activity.

The third semisynthetic bovine AMPA analogue is the one where a norleucine residue is substituted for Gln^4. [Nle^4]-AMPA possesses about 25% of the catalytic activity of native AMPA toward monomeric [2,3-bis(hexanoylthio)propyl]-phosphocholine (Table I). However, [Nle^4]AMPA has completely lost the ability to bind to micelles of the substrate analogue *cis*-9-octadecenylphosphocholine and consequently has no enzymatic activity on micellar substrates like 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine or egg yolk lecithin (Table I). From these results it can be concluded that

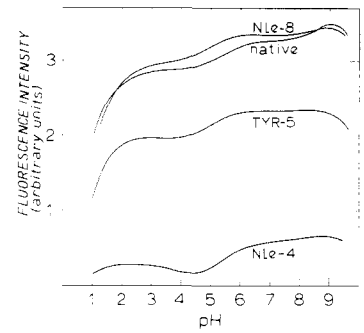

FIGURE 5: Effect of pH on fluorescence intensities (arbitrary units) of native, Nle^8-, Nle^4-, and Tyr^5-substituted ϵ-amidinated bovine phospholipases A_2.

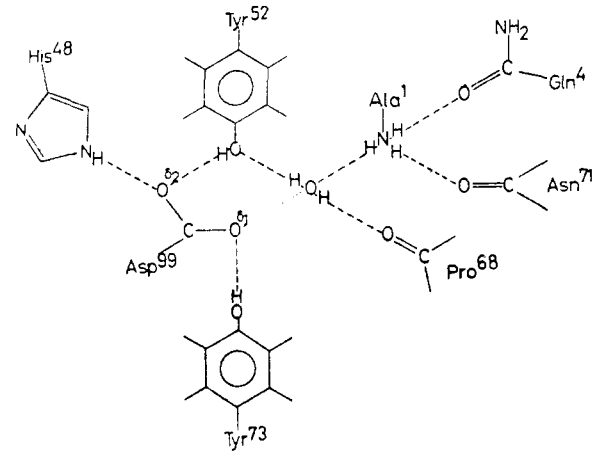

FIGURE 6: Schematic drawing of the interactions between the N terminus and the active site (Dijkstra et al., 1981).

[Nle^4]AMPA does not possess a functional interface recognition site but that its catalytic site is still reasonably intact. In good agreement with these results it was found that [Nle^4]AMPA has only about 25% of the antigenic response of that of native AMPA in the RIA and approaches almost the inertness of des(Ala^1–Gly^7)AMPA in this assay (Figure 3). Moreover, the absence of aromatic resonances originating from Trp^3 in the photo-CIDNP ^{1}H NMR spectrum (Figure 4, upper curve) further supports the conclusion that [Nle^4]-AMPA is devoid of a functional IRS. In the photo-CIDNP spectrum of [Nle^4]AMPA, a new strong Tyr resonance appears which is tentatively assigned to Tyr^{75} (M. R. Egmond, unpublished experiments). The most likely explanation for the absence of Trp^3 CIDNP signals in [Nle^4]AMPA is a more inward position of Trp^3 in the protein, becoming inaccessible for the dye. Instead the dye now strongly reacts with a Tyr residue which was shielded originally from the solvent by Trp^3 in the native proteins. The photo-CIDNP spectrum of [Nle^4]AMPA points to a rather drastic conformational change of the N-terminal region. The effect of this conformational change is also clearly seen in the intensity of the fluorescence of Trp^3 in [Nle^4]AMPA as compared to that of native AMPA (Figure 5). The [Nle^4]AMPA exhibits a much lower fluorescence intensity than the other bovine AMPA's, suggesting a different, more quenching environment of Trp^3 in [Nle^4]AMPA than in native AMPA. From the 1.7-Å X-ray structure of bovine phospholipase A_2, it has been found that the N-terminal amino acids 1–13 form an α-helical structure. An extended system of H bridges was proposed between Ala^1 and Gln^4 and between Ala^1 and the catalytic site Asp^{99} via a H_2O molecule as shown in Figure 6 (Dijkstra et al., 1981). As mentioned before, binding of pancreatic phospholipase A_2

to neutral lipid–water interfaces requires the presence of a free α-NH_2 group of Ala[1]. Apparently the inability of Nle[4] (II)

$$CH_3CH_2CH_2CH_2CH(NH_3^+)COO^-$$
II

$$H_2NCOCH_2CH_2CH(NH_3^+)COO^-$$
III

lacking the carbonyl function of Gln (III) to interact with the α-NH_3^+ group also prevents the formation of the H-bonded system and consequently prevents the formation of a functional IRS. Probably the connection between the α-NH_3^+ group and the active-site Asp[99] via a H_2O molecule is disrupted when Nle is substituted for Gln[4], thereby affecting the catalytic site. This may explain the decreased activity of bovine [Nle[4]]AMPA on monomeric substrate as compared to native bovine AMPA.

Acknowledgments

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Supplementary Material Available

Amino acid analyses of the peptides *N-t*-Boc-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly, *N-t*-Boc-Ala-Leu-Trp(For)-Gln-Tyr-Asn-Gly, and *N-t*-Boc-Ala-Leu-Trp(For)-Nle-Phe-Asn-Gly and the automated sequence analyses of the N terminus of the semisynthetic bovine [Nle[4]]-, [Tyr[5]]-, and [Nle[8]]AMPA and native AMPA (2 pages). Ordering information is given on any current masthead page.

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Interaction of *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ with Ribose and Deoxyribose Diguanosine Phosphates†

Jean-Pierre Girault, Geneviève Chottard, Jean-Yves Lallemand, and Jean-Claude Chottard*

ABSTRACT: The three diguanosine phosphates GpG (4×10^{-4} M), d(GpG) (10^{-5} M), and d(pGpG) (10^{-5} M) have been reacted with *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ (1 Pt/dinucleotide) in water at pH 5.5 and 37 °C. In each case a single product is formed. The three complexes have been characterized by proton nuclear magnetic resonance (^{1}H NMR) and circular dichroism (CD) analyses. They are N(7)–N(7) chelates of the metal with an anti–anti configuration of the bases. They present a conformational change upon deprotonation of guanine N(1)H whose pK_a is ca. 8.7 (D_2O). Their CD spectra, compared to those of the free dinucleotides, exhibit an increase of ellipticity in the 275-nm region, which can be qualitatively related to the characteristic increase reported for platinated DNA and poly(dG)·poly(dC). These results are in favor of the hypothesis of intrastrand cross-linking of adjacent guanines, by the *cis*-$Pt^{II}(NH_3)_2$ moiety, after a local denaturation of DNA.

The mechanism of action of the antitumor *cis*-dichlorodiammineplatinum(II) complex is still the subject of investigations (Rosenberg, 1978). In the cell the active forms of the complex imply the aquation of one or both chloride ligands (Johnson et al., 1980). There is much evidence that suggests that DNA is the primary target of the platinum(II) drug (Roberts & Thomson, 1979). So that the antineoplastic activity of the *cis*-dichlorodiammineplatinum complex, compared to the inactivity of the trans isomer, can be explained, several hypotheses have been put forward that imply a bifunctional coordination of the *cis*-$Pt^{II}(NH_3)_2$ moiety (Roberts & Thomson, 1979). One of them proposes an intrastrand cross-linking of two adjacent guanines [Goodgame et al., 1975; Kelman & Peresie (1979) and previous work cited therein]. Platinum chelation by a d(GpG) sequence has been invoked to account for the gel electrophoretic pattern obtained after *Bam*H1 digestion of platinated DNA (Kelman & Buchbinder, 1978). Such a chelation is supported by the selective inhibition of the restriction endonuclease cleavage of platinated pSM1 DNA, at the cutting site which is adjacent to a $(dG)_4(dC)_4$ sequence (Cohen et al., 1980; Lippard, 1980).

We previously reported that ribose diinosine monophosphate (IpI)[1] (Chottard et al., 1978) and ribose diguanosine monophosphate (GpG)[1] (Chottard et al., 1980) give a single N(7)–N(7)-chelated complex upon reaction with *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$. In this paper we present the results of a comparative study of the binding of the *cis*-$Pt^{II}(NH_3)_2$ moiety to the three ribose and deoxyribose diguanosine phosphates: GpG, d(GpG), and d(pGpG).[1] These results show the generality of facile N(7)–N(7) platinum chelation by diguanosine phosphates and establish the configuration of the bases in the complexes formed. The CD[1] characteristics of these models for intrastrand cross-linking of adjacent guanines are compared with those of platinated DNA [Srivastava et al. (1978) and previous work cited therein; Macquet & Butour, 1978].

Materials and Methods

GpG, d(GpG), and d(pGpG) were used as ammonium salts. The experimental conditions for the reaction between the dinucleotides and *cis*-$[Pt(NH_3)_2(H_2O)_2](NO_3)_2$ have been described previously (Chottard et al., 1980). The stoichiometric reactions (1 Pt/dinucleotide) were run at 4×10^{-4} M concentration for GpG and 10^{-5} M for d(GpG) and d(pGpG) at pH 5.5 and 37 °C. The analytical procedures have also been

† From the Laboratoire de Chimie, Ecole Normale Supérieure, 75231 Paris Cedex 05, France (J.-P.G., J.-Y.L., and J.-C.C.), and the Département de Recherches Physiques, Université Pierre et Marie Curie, 75230 Paris Cedex 05, France (G.C.). *Received June 17, 1981; revised manuscript received October 2, 1981.*

[1] Abbreviations: in dinucleoside monophosphates and dinucleotides, G and I represent guanosine and inosine; p to the left of a nucleoside symbol indicates a 5′-phosphate, and to the right it indicates a 3′-phosphate; H(8)-5′, H(8) proton of the 5′ guanine (at the 5′ end) of GpG; the dinucleoside monophosphates GpG, d(GpG), and IpI will be occasionally referred to as dinucleotides; Guo, guanosine; Ino, inosine; 5′-GMP, guanosine 5′-monophosphate; 5′-IMP, inosine 5′-monophosphate; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; s, singlet; d, doublet; CD, circular dichroism; en, ethylenediamine; tn, trimethylenediamine; dien, diethylenetriamine; $NaDodSO_4$, sodium dodecyl sulfate.

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