Roth, R. I., Jackson, R. L., Pownall, H. J., & Gotto, A. M., Jr. (1977) *Biochemistry 16*, 5030.
Schaefer, E. J., Eisenberg, S., & Levy, R. I. (1978) *J. Lipid Res. 19*, 667.
Segrest, J. P., Jackson, R. L., Morrisett, J. D., & Gotto, A. M. (1974) *FEBS Lett. 38*, 247.
Smith, L. C., Pownall, H. J., & Gotto, A. M. (1978) *Annu. Rev. Biochem. 47*, 751.
Tall, A. R., & Lange, Y. (1978) *Biochim. Biophys. Acta 513*, 185.
Tall, A. R., Small, D. M., Shipley, G. G., & Lees, R. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4940.
Tall, A. R., Shipley, G. G., & Small, D. M. (1976) *J. Biol. Chem. 251*, 3749.
Tall, A. R., Small, D. M., Deckelbaum, R. J., & Shipley, G. G. (1977a), *J. Biol. Chem. 252*, 4701.
Tall, A. R., Deckelbaum, R. J., Small, D. M., & Shipley, G. G. (1977b) *Biochim. Biophys. Acta 487*, 145.
Teng, T., Barbeau, D. L., & Scanu, A. M. (1978) *Biochemistry 17*, 17.
Zimmer, S., & Biltonen, R. (1972) *J. Solution Chem. 1*, 291.

Semisynthesis of Phospholipase A_2. Preparation and Properties of Arginine-6 Bovine Pancreatic Phospholipase A_2†

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

ABSTRACT: The major differences between porcine and bovine pancreatic phospholipases A_2 are the low affinity of the bovine enzyme for lipid–water interfaces and its low capacity to penetrate more densely packed monolayers of lecithins. In the proposed binding site for lipid–water interfaces the porcine enzyme has an Arg residue at position 6 which is Asn in the bovine enzyme. In order to study whether this difference affects the above-mentioned properties, a hybrid bovine phospholipase A_2 that has Arg at position 6 was prepared. Bovine pancreatic prophospholipase A_2 was converted into the fully ϵ-amidinated zymogen (AMPREC) which produced enzymatically active ϵ-amidinated phospholipase A_2 (AMPA) upon limited proteolysis. CNBr cleavage of AMPREC at the unique Met residue at position 8 gave des(Ala^1–Met^8)AMPA, a protein completely devoid of all enzymatic activity. Met^8 was reintroduced by coupling of the latter protein with Boc-Met-*N*-hydroxysuccinimide ester followed by treatment with trifluoroacetic acid, yielding des(Ala^1–Gly^7)AMPA. Subsequently Boc-Ala-Leu-Trp(For)-Gln-Phe-Arg-Gly, synthesized by the solid-phase technique, was coupled by using the mixed-anhydride method. Removal of the protecting groups and purification gave semisynthetic bovine [Arg^6]AMPA in 30% yield. The feasibility of this procedure was proven unambiguously by the retroconversion of des(Ala^1–Met^8)AMPA into the original bovine AMPA, being identical in all respects including enzymatic activity with the starting AMPA. Both the affinity of bovine [Arg^6]AMPA for lipid–water interfaces and its ability to penetrate more densely packed monolayers of lecithin are considerably increased as compared to the bovine AMPA. In these respects bovine [Arg^6]AMPA was found to be almost identical with the porcine AMPA. Moreover, bovine [Arg^6]AMPA possesses enhanced enzymatic activity as compared to bovine and porcine AMPA. It can be concluded that substitution of Asn^6 by Arg in bovine phospholipase A_2 improves the binding for lipid–water interfaces. The concomitant increase in enzymatic activity strongly suggests an effect of the lipid binding site on the active site.

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 pancreatic enzyme occurs as a zymogen, prophospholipase A_2, which is converted into the active enzyme by splitting the N-terminal heptapeptide (Figure 1) upon limited proteolysis (de Haas et al., 1968). Both proteins have low, though comparable, activities toward monomeric substrate, indicating the presence of a functionally active site in the zymogen as well (Pieterson et al., 1974; Volwerk et al., 1979). When substrate is present as a certain organized lipid–water interface, like, e.g., micelles, there is a large increase in enzymatic activity of phospholipase A_2 but not for its zymogen (Pieterson et al., 1974). Various direct binding studies with micellar substrate analogues demonstrated that phospholipase A_2, in contrast to the zymogen, binds to these lipid–water interfaces (Pieterson et al., 1974; van Dam-Mieras et al., 1975; Soares de Araujo et al., 1979). Therefore, the pancreatic phospholipases A_2 were proposed to possess a specific binding site for lipid–water interfaces called the interface recognition site (IRS).[1] A conformational change

† From the Laboratory of Biochemistry, State University of Utrecht, Transitorium 3, University Center "De Uithof", Padualaan 8, 3508 TB Utrecht, The Netherlands. *Received August 15, 1980.* 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.).

‡ The key concepts of the present paper were presented at the 16th European Peptide Symposium, Helsingør (Denmark), Aug 31–Sept 6, 1980.

[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (*Biochemistry 6*, 362, 3287 (1967); *11*, 1726 (1972)) were used throughout. Other abbreviations used: AMPA, ϵ-amidinated phospholipase A_2; AMPREC, ϵ-amidinated prophospholipase A_2; tosyl, *p*-toluenesulfonyl; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; For, formyl; LC, liquid chromatography; iBuOCOCl, isobutyl chloroformate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; IRS, interface recognition site; TFA, trifluoroacetic acid; CPK model, Corey, Pauling, and Koltun space-filling molecular model; CIDNP, chemically induced dynamic nuclear polarization; TSP, [2,2,3,3-$^2H_4$]trimethylsilylpropionate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; cmc, critical micellar concentration; EtOH, ethanol; DIEA, *N*,*N*′-diisopropylethylamine; DMF, dimethylformamide; NMR, nuclear magnetic resonance; pH*, uncorrected pH meter readings in ^2H_2O; ppm, parts per million.

0006-2960/81/0420-1584$01.25/0

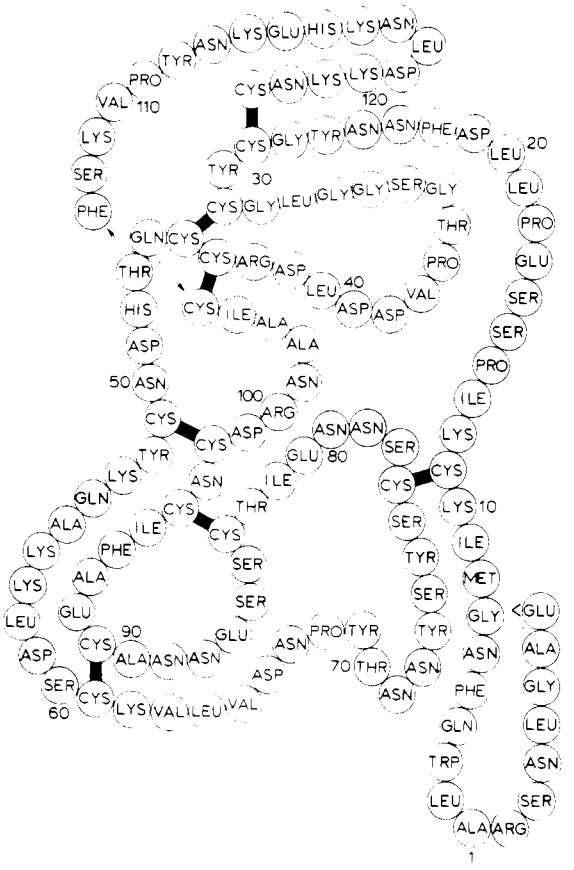

FIGURE 1: Primary structure of bovine pancreatic prophospholipase A_2.

occurs upon binding, giving rise to an optimization of the active site (Verger et al., 1973; Pieterson et al., 1974). Previously it was demonstrated by selective chemical modification of the N-terminal α-NH_2 group of Ala^1 in porcine pancreatic phospholipase A_2 that a free α-NH_2 group is absolutely required for the enzyme to bind to neutral lipid–water interfaces and to display its full enzymatic activity on micellar substrates (van Dam-Mieras et al., 1975; Slotboom & de Haas, 1975; Slotboom et al., 1978). Subsequent studies revealed that in the porcine phospholipase A_2 Trp^3, Arg^6, Tyr^{69}, Leu^{19} (= Tyr^{19} in the horse phospholipase A_2), and Met^{20} partly constitute the IRS (van Dam-Mieras et al., 1975; Meijer et al., 1979; Meijer, 1979). Inspection of the CPK model built according to the 1.7-Å X-ray coordinates from bovine phospholipase A_2 shows that the IRS residues are close together, forming a hydrophobic edge surrounding the cavity where the active site (His^{48}, Asp^{49} and Ca^{2+}, Asp^{99}) is located and that the α-NH_2 group is connected via a H_2O molecule to Asp^{99} (Dijkstra et al., 1978; Dijkstra, 1980; Verheij et al., 1980) (Figure 6). Recently evidence was presented from isothermal calorimetry that the binding of the porcine phospholipase A_2 to micelles is predominantly due to hydrophobic interaction (Soares de Araujo et al., 1979).

Although both bovine and porcine pancreatic phospholipases A_2 have comparable enzymatic activities on micellar and monomeric substrates, it is known that the affinity of the bovine enzyme for neutral lipid–water interfaces is much weaker than that of the porcine enzyme (Dutilh et al., 1975). Moreover, the bovine phospholipase A_2 can penetrate monolayers of 1,2-didecanoyl-*sn*-glycero-3-phosphocholine up to 8 dyn/cm whereas the porcine enzyme penetrates these films up to 12 dyn/cm. Most likely these differences are due to differences in the amino acid composition of the binding sites for lipid–water interfaces of the bovine and porcine phospholipases A_2. In this respect the substitution of Arg at position 6 in the porcine phospholipase A_2 by an Asn in the bovine enzyme could be responsible for the observed differences. The present paper describes the semisynthesis of bovine [Arg^6]AMPA and some of the properties of this hybrid enzyme.

Experimental Section

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 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 and converted into phospholipase A_2 by limited proteolysis as described by Dutilh et al. (1975). Porcine pancreatic prophospholipase A_2 was purified from porcine pancreas and converted into phospholipase A_2 by limited proteolysis as described by Nieuwenhuizen et al. (1974). ϵ-Amidinated porcine phospholipase A_2 was prepared as described previously (Slotboom & de Haas, 1975). *N-t*-Boc derivatives of Gly, L-Asn, L-Ala, L-Leu·H_2O, L-Phe, L-Gln, and N^g-tos-L-Arg were obtained from Vega Biochemicals (Tucson, AR). *N-t*-Boc-N^{in}-formyl-L-Trp was purchased from the Protein Research Foundation (Osaka, Japan) and *N-t*-Boc-L-Met-*N*-hydroxysuccinimide ester from Fluka A.G. (Switzerland). 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, 1,2-ethanedithiol, and 1-hydroxybenzotriazole were from Aldrich (Belgium). Dimethylformamide (Uvasol), 2-chloroethanol, and methylene dichloride (pro analysi) were products of Merck (Germany). Liquid HF was from Matheson (U.S.A.), acetonitrile (high-performance LC quality) from Baker Chemicals (U.S.A.), trifluoroacetic acid from Pierce (U.S.A.), cobalt trifluoride from ICN (U.S.A.), cesium hydrogen carbonate from Alfa Research Chemicals (U.S.A.), and *p*-bromophenacyl bromide from Fluka A.G. (Switzerland). Trypsin and TPCK-treated trypsin were products from Serva (Germany). 1,2-Didecanoyl- and 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine were prepared as described by Cubero Robles & Van den Berg (1969). *cis*-9-Octadecenylphosphocholine and 2,3-bis(hexanoylthio)-propylphosphocholine were synthesized as described by van Dam-Mieras et al. (1975) and Volwerk et al. (1979), respectively. All other chemical used were of the highest purity available.

Methods

Amino acid analyses were performed by the method of Spackman et al. (1958) on a Beckman Unichrom amino acid analyzer equipped with a high-sensitivity attachment, or on a Technicon TSM amino acid analyzer. Samples were hydrolyzed for 24 h at 110 °C in evacuated sealed tubes with 5.8 N HCl. Trp was determined similarly by addition of 4% of thioglycolic acid as described by Matsubara & Sasaki (1969). Protein concentrations were calculated from the absorbance at 280 nm with an E_{1cm} (1%) of 12.3 for the porcine prophospholipase A_2, bovine (pro)phospholipase A_2, and their ϵ-amidinated derivatives and for bovine [Arg^6]- and [Asn^6]-AMPA. A value of 13.0 was used for the porcine phospholipase A_2 and its amidinated analogue. Slab gel electrophoresis

was performed for 1 h at 50 mA on 1.5 mm thick 7.5% polyacrylamide gels at pH 4.3 and 9.6 with the apparatus as described by Studier (1973). The gels and buffer solutions were prepared according to the manufacturer's instructions (Shandon, England). Protein staining was done with a 0.1% solution of amido black in 7% acetic acid for 1 h and destaining in 7% acetic acid overnight. N-Terminal amino acid residues were determined by dansylation according to the method of Gray (1972). Enzyme activities were routinely determined by the titrimetric assay procedure with egg yolk lipoproteins as substrates (Nieuwenhuizen et al., 1974). In contrast to the described procedure a 2-fold higher Ca^{2+} concentration and an 1.5-fold higher sodium deoxycholate concentration were used, while the fatty acids were titrated with standardized 0.02 N NaOH. Kinetic measurements using micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine and monomeric 2,3-bis(hexanoylthio)propylphosphocholine were performed as described before [de Haas et al. (1971) and Volwerk et al. (1979), respectively]. Second-order rate constants (k_2) for the inactivation of porcine and bovine AMPA and bovine [Arg^6]AMPA were determined at pH 6.0 and 30 °C as described by Volwerk et al. (1974) and Volwerk (1979). Ultraviolet difference spectra were obtained at 25 °C with an Aminco DW-2a spectrophotometer with an automatic baseline correction accessory (Midan data analyzer, from Aminco, U.S.A.) as previously described (van Dam-Mieras et al., 1975). Fluorescence spectroscopy was done as described by van Dam-Mieras et al. (1975).

Monolayer Experiments. The zero-order trough with two compartments and the surface barostat technique used to measure substrate hydrolysis were identical with those described by Verger & de Haas (1973). Calculation of the induction time (τ) was performed as previously described by Verger et al. (1973). Surface pressure π was measured by the Wilhelmy plate method, using a thin platinum plate (perimeter 3.94 cm) attached to a Beckman R II C Model LM 600 electromicrobalance.

NMR Spectroscopy. ^{1}H NMR (360-MHz) spectra were obtained with a Bruker HX-360 spectrometer at the SON Facility in Groningen, The Netherlands, using quadrature detection in the pulse Fourier transform. Photo-CIDNP spectra (Kaptein, 1978) were recorded as previously described (Jansen et al., 1978; Egmond et al., 1980) with 3-carboxymethyllumiflavin as the dye. Dioxane was added as internal standard. However, chemical shifts were calculated relative to TSP, using 3.747 ppm for the chemical shift difference between dioxane and TSP.

Preparation of Bovine AMPREC and AMPA. Amidination of bovine prophospholipase A_2 (3 g) was performed as described previously for the porcine zymogen (Slotboom & de Haas, 1975). The crude ε-amidinated bovine prophospholipase A_2 was purified at 4 °C by chromatography on a DEAE-cellulose column (60 × 3 cm) equilibrated with 5 mM Tris (pH 8.0) and developed with 10 L of a linear salt gradient reaching 0.5 M NaCl in the same buffer. Fractions containing ε-amidinated zymogen were pooled, dialyzed against 1% of acetic acid at 4 °C for 24 h, and lyophilized to yield 2.1 g (70%) of electrophoretically homogeneous ε-amidinated bovine prophospholipase A_2. Reaction of the ε-amidinated zymogen with 2,4-dinitrofluorobenzene followed by 5.8 N HCl hydrolysis for 18 h at 110 °C and amino acid analysis on the short column showed the quantitative conversion (>99.9%) of lysyl residues into ε-acetimidolysine residues (Wofsy and Singer, 1963). The amino acid composition of ε-amidinated prophospholipase A_2 was found to be identical with that of the native protein except for the modified lysine residues.

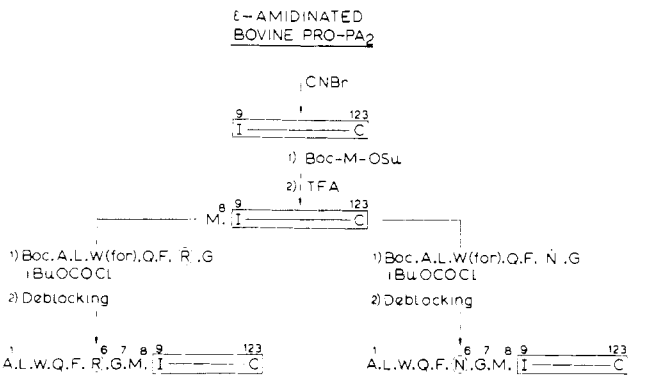

FIGURE 2: Scheme depicting semisynthesis of ε-amidinated bovine [Arg^6]- and [Asn^6] phospholipases A_2. Pro-PA_2, Boc, Boc-M-OSu, For, TFA and iBuOCOCl refer to prophospholipase A_2, *t*-butyloxycarbonyl, *N-tert*-butyloxycarbonyl-L-Met-*N*-hydroxysuccinimide ester, formyl, trifluoroacetic acid, and isobutyl chloroformate, respectively.

ε-Amidinated bovine phospholipase A_2 was obtained by limited tryptic proteolysis of ε-amidinated bovine prophospholipase A_2 (850 mg) as described before (Slotboom & de Haas, 1975). Purification of the ε-amidinated phospholipase A_2 was performed at 4 °C on a CM-cellulose column (3 × 37 cm) equilibrated with 5 mM sodium acetate (pH 6.0) and developed with 2 L of a linear salt gradient reaching 0.2 M NaCl in the same buffer. Fractions containing AMPA were pooled, dialyzed for 24 h at 4 °C, and lyophilized, yielding 623 mg of pure AMPA (73%). The amino acid composition[2] of AMPA was found to be identical with that of the native phospholipase A_2 except for the modified lysine residues. End group analysis using the dansyl procedure showed the presence of only Ala.

Preparation of Bovine Des(Ala^1–Gly^7)AMPA. Bovine des(Ala^1–Met^8)AMPA was prepared by reacting ε-amidinated prophospholipase A_2 (1.2 g) dissolved in 70% of formic acid (240 mL) with CNBr (1.4 g) in the dark for 16 h at room temperature (Gross & Witkop, 1962) (Figure 2). Removal of most of the formic acid was done by evaporation in vacuo at room temperature. The resulting solution was diluted with distilled water and lyophilized. After removal of the N-terminal pentadecapeptide by dialysis, the des(Ala^1–Met^8)AMPA was purified by chromatography on CM-cellulose and DEAE-cellulose by using the above-described conditions. After the protein peak containing the des(Ala^1–Met^8)AMPA was pooled, followed by dialysis and lyophilization, 1.01 g of electrophoretically pure des(Ala^1–Met^8)AMPA was obtained (yield 95%). Dansylation showed Ile to be the only N-terminal amino acid residue. Trp was found to be absent as determined by amino acid analysis and fluorescence spectroscopy. A small amount of the latter protein was incubated at pH 8.0 with trypsin in order to check whether unreacted AMPREC was still present in the des(Ala^1–Met^8)AMPA. No enzymatic activity could be detected, not even after repeated additions of fresh trypsin, indicating that no unreacted AMPREC was present.

Bovine des(Ala^1–Gly^7)AMPA was prepared by coupling of des(Ala^1–Met^8)AMPA (0.6 g) with *N-t*-Boc-L-Met-*N*-hydroxysuccinimide ester as described previously (Slotboom & de Haas, 1975) (Figure 2); 0.47 g of des(Ala^1–Gly^7)AMPA was obtained after purification on CM-cellulose as described

[2] The amino acid analyses of all proteins and peptides described in this paper were found to be in good agreement with the expected values and were submitted to the reviewers for examination.

above for bovine AMPA (yield 78%). The material was pure on gel electrophoresis at pH 9.6 and had a correct amino acid composition. End group analysis with the dansyl procedure gave only Met.

Synthesis of Peptides (t-Boc-Ala-Leu-Trp(For)-Gln-Phe-Arg-Gly and t-Boc-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly). Peptides were synthesized by the solid-phase method (Erickson & Merrifield, 1976) by using a computerized peptide synthesizer (SYN-1) developed by the Danish Institute of Protein Chemistry (Villemoes et al., 1976, 1978). *N-t*-Boc-glycine was esterified to chloromethylated polystyrene resin 1% cross-linked with divinylbenzene (S-XI, Bio-Rad, U.S.A.; 1.34 mmol of Cl/g) according to the method of Gisin (1973). The degree of substitution was determined by amino acid analysis by using the procedure of Westall et al. (1972) and found to be 1.17 mmol of Boc-Gly/g of resin. The synthesis consisted of stepwise double couplings with dicylclohexylcarbodiimide-activated Boc amino acids according to the following automated protocol: (1) CH_2Cl_2 5 times for 3 min, (2) EtOH 2 times for 3 min, (3) CH_2Cl_2 5 times for 3 min, (4) 50% TFA/CH_2Cl_2 for 3 min, (5) 50% TFA/CH_2Cl_2 for 20 min, (6) CH_2Cl_2 6 times for 3 min, (7) EtOH 2 times for 3 min, (8) CH_2Cl_2 5 times for 3 min, (9) 5% DIEA/CH_2Cl_2 for 5 min, (10) CH_2Cl_2 3 times for 3 min, (11) EtOH 2 times for 3 min, (12) CH_2Cl_2 3 times for 3 min, (13) 5% DIEA/CH_2Cl_2 for 5 min, (14) CH_2Cl_2 5 times for 3 min, (15) 3 equiv of Boc-amino acid/CH_2Cl_2 for 10 min, then 3 equiv of DCC/CH_2Cl_2 for 120 min, (16) CH_2Cl_2 5 times for 3 min, (17) EtOH 2 times for 3 min, (18) CH_2Cl_2 3 times for 3 min, (19) EtOH 2 times for 3 min, (20) CH_2Cl_2 5 times for 3 min, and (21) repeat steps 9–21. For prevention of diketopiperazine formation (Gisin & Merrifield, 1972) during amino acid coupling to form the tripeptide, the sequence of addition of DCC and Boc-amino acid was reversed (operation 15). Since Boc-Asn and Boc-Gln were coupled as the hydroxybenzotriazole esters (Hruby et al., 1973), the automated procedure was interrupted before these coupling steps. After step 14, 3 equiv of DCC and 1-hydroxybenzotriazole in DMF was mixed at 0 °C, then 3 equiv of Boc-amino acid in DMF was added, and the resulting solution was immediately added manually to the reaction vessel. After 3 h of reaction with stirring, the automatic mode was then continued by step 16. Tryptophan was protected by a formyl group and arginine with the tosyl moiety. After incorporation of Trp, deblocking (steps 4 and 5) was performed in the presence of 5% 1,2-ethanedithiol, preventing oxidation of tryptophan.

Peptides were removed from the resin (1.09 g) by liquid HF, dried over cobalt trifluoride, in the presence of anisole (10%) by using an HF-Reaction apparatus type I (Peptide Institute, Inc. Osaka, Japan) for 30 min at 0 °C. After evaporation of the HF in vacuo, anisole was removed by extraction with ethyl acetate (3 times, 10 mL). Peptides were successively extracted with 10 mL of acetic acid, acetic acid–H_2O (1:1 v/v), and water (4 times). The combined extracts were diluted with excess distilled water and lyophilized. The yield of the crude peptides was about 80% based on the amount of Boc-Gly-resin.

Purification of Peptides. Purification of peptides was performed on a chromobeads type P (Technicon) column (0.9 × 50 cm) at 55 °C and 560 psi with a flow rate of 1 mL/min with a Technicon TSM amino acid analyzer. The peptides were eluted with a linear pH gradient obtained from 200 mL of 0.2 M pyridine–acetate (pH 3.1) and 200 mL of 2 M pyridine–acetate (pH 5.0) (Schroeder, 1967). Further purification of the peptides was done by high-performance LC on a C_{18} reversed-phase Lichrosorb column (1 × 25 cm, particle size 10 μm, Altex, U.S.A.) by using a Kontron LC Pump 410 (Kontron, Switzerland) (Figure 3). The high-performance LC column was equilibrated with 10% acetonitrile in water containing 0.1% trifluoroacetic acid at room temperature. Peptides were injected by using a Rheodyne 7125 in portions of 10 mg dissolved in 1 mL of 40% acetonitrile in water containing 0.1% trifluoroacetic acid. Elution of the peptides was performed with a linear gradient from 10 to 40% acetonitrile in water containing 0.1% trifluoroacetic acid at a flow rate of 6 mL/min. Detection was at 220 nm with Kontron Uvikon LCP 725 (Kontron, Switzerland). The pure peptides were obtained in a yield of 70% based on the crude material after HF cleavage. The α-amino group of the purified peptides was reacted with di-*tert*-butyl dicarbonate according to the procedure of Moroder et al. (1976). The resulting *t*-Boc protected peptides were again purified by high-performance LC as described above, now by using a linear gradient reaching 90% of acetonitrile in water containing 0.1% trifluoroacetic acid. *t*-Boc-Ala-Leu-Trp(For)-Gln-Phe-Arg-Gly and *t*-Boc-Ala-Leu-Trp(For)-Gln-Phe-Asn-Gly were almost quantitatively obtained in a pure state and showed the correct amino acid compositions.

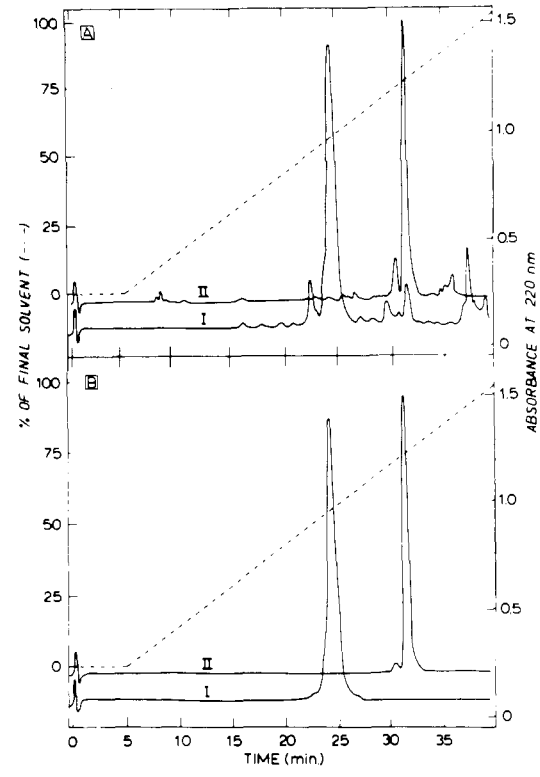

FIGURE 3: Analytical high-performance LC chromatograms obtained with the peptides Ala[1]-Leu-Trp(For)-Gln-Phe-Arg[6]-Gly[7] (I) and Ala[1]-Leu-Trp(For)-Gln-Phe-Asn[6]-Gly[7] (II) before (A) and after (B) preparative purification. Conditions as described under Methods.

Preparation of Semisynthetic Bovine [Asn[6]]- and [Arg[6]]-AMPA. The α-amino-protected peptides were condensed to des(Ala[1]–Gly[7])AMPA by using the mixed anhydride method as described by Naithani (1979) with some slight modifications (Figure 3). To a solution of *N-t*-Boc-Ala[1]-Leu-Trp(For)-Gln-Phe-Asn[6]-Gly[7] or *N-t*-Boc-Ala[1]-Leu-Trp(For)-Gln-Phe-Arg[6]-Gly[7] (40 μmol) and *N*-methylmorpholine (40 μmol) in 20 mL of DMF at −15 °C was added isobutyl chloroformate (38 μmol). After being stirred for 5 min, this mixture was added to a solution of des(Ala[1]–Gly[7])AMPA (8 μmol) in 3 mL of 0.1 M Hepes buffer (pH 7.0) at 4 °C. The reaction mixture was stirred for 20 min at 4 °C and 20 min at room temperature. The reaction was stopped by addition of 10 mL of a 3 M hydroxylamine solution (pH 7.0) and stirring was

continued for another 20 min. The protein was desalted on a Sephadex G-25 coarse column (3 × 80 cm) equilibrated with 1% acetic acid, and the fractions containing the protein peak were pooled and lyophilized. The crude *N-t*-Boc AMPAs were then deformylated by treatment with 10 mL of a 1 M $NaHCO_3$ solution (pH 9.0) for 24 h as described by Yamashiro & Li (1973). After deformylation, the semisynthetic *N-t*-Boc-AMPAs were desalted on a Sephadex G-25 coarse column (3 × 80 cm) in 1% acetic acid, lyophilized, and purified by chromatography on CM-cellulose at pH 6.0 as described above for AMPA. Fractions containing *N-t*-Boc-[Arg^6]AMPA or *N-t*-Boc-[Asn^6]AMPA were pooled, lyophilized, and treated with 2 mL of anhydrous trifluoroacetic acid under N_2 at room temperature. After 15 min the mixture was diluted with a large excess of cold water and lyophilized immediately. The semisynthetic AMPA analogues obtained were purified on DEAE-cellulose as described above for AMPREC. Fractions containing the pure [Arg^6]- or [Asn^6]AMPAs, respectively, were pooled, dialyzed, and lyophilized. The semisynthetic [Arg^6]- and [Asn^6]AMPAs, which were obtained in 30% yield, were homogeneous on gel electrophoresis (pH 4.3 and 9.6) and had the expected amino acid compositions.

Results and Discussion

Semisynthesis of proteins is an attractive approach to explore the role of essential amino acid residues (Chaiken & Komoriya, 1978). By use of this method analogues have been prepared for insulin, cytochrome *c*, myoglobin, ribonuclease, trypsin inhibitor, and phospholipase A_2 [see reports in Offord & DiBello (1978)]. Substitution of the N-terminal amino acid of ϵ-amidinated porcine phospholipase A_2 clearly showed the importance of Ala^1 for enzymatic activity on micellar substrates (Slotboom & de Haas, 1975). These specific amino acid substitutions were done by removing the N-terminal amino acid using the Edman procedure and subsequent replacement by the desired activated N-protected amino acid derivatives. The stepwise removal of N-terminal amino acid residues by consecutive Edman cycles on a preparative scale is, however, limited to the first three or four amino acid residues, mainly because of the rapidly diminishing yield as a consequence of the required purifications. If an amino acid residue which is located further away from the N terminus, has to be substituted, it is advantageous to selectively split peptides from the N-terminal site. Obviously, the success of such an approach depends on the possibility of a specific chemical or enzymatic cleavage of a single, conveniently located peptide bond. Fortunately, bovine prophospholipase A_2 possesses only one Met residue at position 8 (Figure 1), enabling specific chemical cleavage with CNBr. In order to prevent later on undesired coupling of peptide fragments to the ϵ-amino groups of Lys residues, the protein had first to be protected. From our previous experience with porcine phospholipase A_2 it also turned out to be advantageous for the present semisynthesis of bovine phospholipase A_2 to use the ϵ-amidinated enzyme. Complete amidination of bovine prophospholipase A_2 followed by limited proteolysis gave ϵ-amidinated bovine phospholipase A_2 (AMPA), an enzyme possessing about 75% of the enzymatic activity of the native enzyme: V_{max} values are 2300 and 3000 μequiv min^{-1} (mg of protein)$^{-1}$, respectively.[3] Also with respect to substrate and Ca^{2+} binding as well as their behavior on monolayers both enzymes were found to be almost identical. Therefore, it is not necessary to deblock the ϵ-acetimidolysine residues to study the effect of amino acid substitutions. Thus, CNBr fragmentation was done on the ϵ-amidinated prophospholipase A_2, producing des(Ala^1–Met^8)AMPA (Figure 2). This protein, completely devoid of all its enzymatic activity, consists of one single polypeptide chain with all seven disulfide bridges intact and has only one free NH_2 group, viz., the α-NH_2 group. Noncovalent recombination of the N-terminal octapeptide with the protein entity did not lead to restoration of enzymatic activity, therefore necessitating their covalent introduction. N-protected peptides which do not contain free carboxyl groups other than the C-terminal one can most conveniently be coupled to the α-NH_2 group of protein fragments having blocked ϵ-amino groups by the mixed carbonic anhydride method (Meienhofer, 1979). A major advantage of the mixed carbonic anhydride method is that the acylation can be conducted in aqueous organic solutions of the required composition to keep both the hydrophobic peptide and the protein fragment in solution. Other attractive features of the mixed carbonic anhydride method known are the high reactivity, the comparatively high purity of the products, and in general the satisfactory yields (Meienhofer, 1979). The major disadvantage of the mixed carbonic anhydride method has always been its high tendency to give rise to racemization. Although Anderson et al. (1967) gave well-defined conditions for mixed carbonic anhydride couplings with a minimum risk of racemization, it appeared to be better to prepare the bovine AMPA analogue without risk of racemization. Therefore, as shown in Figure 2, bovine des(Ala^1–Met^8)AMPA was converted into bovine des(Ala^1–Gly^7)AMPA by coupling with *t*-Boc-L-Met-*N*-hydroxysuccinimide ester followed by deprotection with TFA. The advantage of this approach is that the desired *N*-protected peptides with C-terminal Gly can be covalently linked to the protein fragment by use of the mixed carbonic anhydride method. For this purpose two heptapeptides with Gly as C terminus were synthesized, one with the original N-terminal sequence, viz., Ala^1-Leu-Trp(For)-Gln-Phe-Asn-Gly^7, and one with the Asn^6 substituted by an Arg residue. These peptides were synthesized by the solid-phase method and cleaved from the solid support by liquid hydrogen fluoride without problems. The crude peptides obtained were then purified to homogeneity by ion-exchange chromatography and high-performance LC (Figure 3). As shown in Figure 2 these purified peptides were N-protected with the *t*-Boc group and coupled to the bovine des(Ala^1–Met^8)AMPA by the mixed carbonic anhydride method as discussed above. After deprotection and purification, semisynthetic bovine [Arg^6]AMPA and [Asn^6]AMPA were obtained in about 30% yield. The purpose of preparing bovine [Asn^6]AMPA was to show the feasibility of the applied procedures. As can be seen from Table I and Figure 4, semisynthetic bovine AMPA behaves almost completely identically with the original bovine AMPA, including the recovery of full enzymatic activity by using different assays. It can thus be concluded that the observed differences of bovine [Arg^6]AMPA and the original bovine AMPA can be fully attributed to the substitution of Asn^6 by Arg.

As demonstrated previously (van Dam-Mieras et al., 1975), trypsin selectively splits the Arg^6–Ser^7 linkage in porcine AMPA with a concomitant loss of enzymatic activity. This splitting is nearly completely prevented by the simultaneous presence of micellar substrate analogues and Ca^{2+} ions. Mainly on account of this observation Arg has been assigned to be part of the IRS. From Table I it is clear that the binding of bovine [Arg^6]AMPA to micelles of the substrate analogue

[3] V_{max} values were determined on micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine as described in the legends of Table I.

Table I: Comparison of Various Properties of Semisynthetic Bovine ε-Amidinated [Asn⁶]- and [Arg⁶]phospholipases A_2 with ε-Amidinated Bovine and Porcine Phospholipases A_2

	bovine			porcine
	AMPA	[Asn⁶]AMPA	[Arg⁶]AMPA	AMPA
sp act.[a] [μequiv min^{-1} (mg of protein)$^{-1}$] (egg yolk assay)	50 ± 3	46 ± 3	120 ± 6	400 ± 20
V_{max}[b] [μequiv min^{-1} (mg of protein)$^{-1}$] (micellar L-dioctanoyllecithin)	2300 ± 160	2500 ± 50	6300 ± 300	2000 ± 40
k_{cat}/K_M[c] (s^{-1} M^{-1}) (monomeric bis(thiohexanoyl)lecithin)	172 ± 10	179 ± 10	972 ± 50	555 ± 30
$K_{Ca^{2+}}$[d] (mM)	4.61 ± 0.08		4.42 ± 0.13	2.10 ± 0.02
K_D[e] (mM) (binding of micellar *cis*-9-octadecenylphosphocholine)	5.0 ± 0.6	5.4 ± 0.6	0.40 ± 0.05	0.16 ± 0.02
k_2[f] (M^{-1} min^{-1}) (second-order rate constant for inactivation with *p*-bromophenacyl bromide)	2300 ± 230		2760 ± 250	205 ± 20

[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., 1974). Conditions used: 50 mM acetate, 0.1 M NaCl, pH 6.0, 25 °C. [e] Determined by ultraviolet difference spectroscopy (van Dam-Mieras et al., 1975). Conditions used: 50 mM acetate, 0.1 M $CaCl_2$, 0.1 M NaCl, pH 6.0, 25 °C. [f] Determined at pH 6.0 and 30 °C as described by Volwerk et al. (1974) and Volwerk (1979).

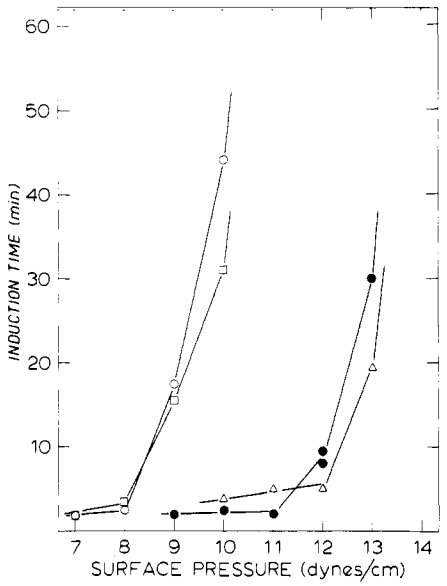

FIGURE 4: Influence of surface pressure of a 1,2-didecanoyl-*sn*-glycero-3-phosphocholine monolayer on the induction time of the kinetics of hydrolysis by bovine AMPA (○), semisynthetic bovine [Asn⁶]AMPA (□), semisynthetic bovine [Arg⁶]AMPA (●), and porcine AMPA (△). Conditions: Tris–acetate buffer (10 mM), pH 6.0, $CaCl_2$ (0.02 M), NaCl (0.1 M), 25 °C.

cis-9-octadecenylphosphocholine greatly improves as compared to that of bovine AMPA and approaches almost that of porcine AMPA. Figure 4 shows the ability of bovine [Arg⁶]AMPA as compared to porcine and bovine AMPA to penetrate into monolayers of 1,2-didecanoyl-*sn*-glycero-3-phosphocholine as a function of pressure. It is obvious from this figure that the rather weak penetration capacity of bovine AMPA is considerably improved by substitution of Asn⁶ by Arg⁶: the bovine [Arg⁶]AMPA penetrates films up to 12 dyn/cm just as the porcine AMPA does. Both the affinity for lipid–water interfaces and the penetration capacity give information about the properties of the lipid binding site (IRS) of the enzyme. From these results it is clear that the substitution of Asn⁶ by Arg in the bovine AMPA considerably improves the IRS. Comparable effects were also recently observed by Meijer et al. (1979). Introduction of a dansyl group on NH_2-Tyr¹⁹ in horse phospholipase A_2 causes also a considerable higher affinity for lipid–water interfaces as well as a more powerful penetrating site. In this example, an additional hydrophobic group is introduced, most likely directly improving the hydrophobic properties of the IRS. Taking into account that the binding of the enzyme to micelles is mainly hydrophobic (Soares de Araujo et al., 1979), it is quite obvious that upon extension of the hydrophobic IRS the affinity for lipid–water interfaces is increased. However, upon substitution of an Asn by an Arg residue one would a priori not expect an improvement of the hydrophobic interaction. It is also hard to understand that one extra positive charge, introduced by the Asn → Arg substitution, can account for the considerably increased binding and penetrating capacity toward neutral lipid–water interfaces. A more plausible explanation could be that due to the substitution of Asn⁶ by an Arg residue a conformational change occurs, giving rise to reorientation of certain hydrophobic residues of the IRS in such a way that the affinity for neutral lipid–water interfaces is improved. In agreement with this assumption it was found by ^1H NMR spectroscopy, using photo-CIDNP (Figure 5A,B), that resonances from Trp³ as well as from Tyr⁶⁹ both belonging to the IRS are affected by the substitution of Asn⁶ by an Arg residue in bovine [Arg⁶]AMPA as compared to the bovine AMPA. As a matter of fact, the poorly resolved CIDNP Trp³ signals of bovine [Arg⁶]AMPA more resembled those of the porcine AMPA (Jansen et al., 1978) rather than the well-resolved Trp³ resonances of the bovine AMPA. Unfortunately, for Tyr CIDNP emission lines it is not possible to directly compare these spectra at pH 5 because the porcine enzyme contains an additional tyrosine, Tyr¹²³, which gives a strong emission line at 6.9 ppm. A CIDNP signal from Tyr⁶⁹ in the porcine enzyme is only observed at higher pH values (around pH 9), while at lower pH values its resonance broadens and partially overlaps with the Tyr¹²³ CIDNP signal. Interestingly, the Tyr⁶⁹ emission line in the CIDNP spectrum of bovine [Arg⁶]AMPA (Figure 5B) is broad as compared to that of bovine AMPA, while a lower intensity of its ^1H NMR resonance is apparent as shown in Figure 5B,D (asterisk). Also in the deconvolution spectra the resonances originating from residues Trp³ and Tyr⁶⁹ are perturbed (Figure 5C,D), this can be seen more clearly from the difference spectrum (Figure 5E).

From Table I it can be concluded that the bovine [Arg⁶]-AMPA possesses an enhanced enzymatic activity both on monomeric substrate and on micellar substrate as compared to bovine and porcine AMPA. Taking into account that the

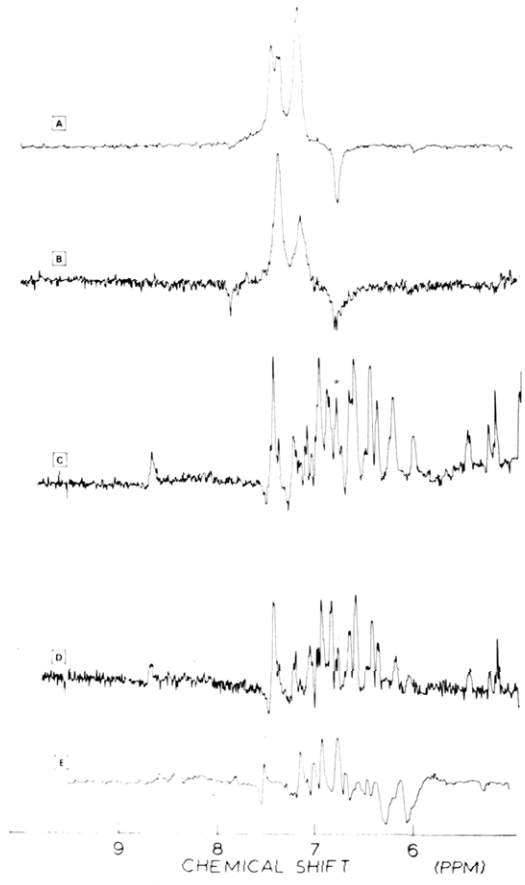

FIGURE 5: 360-MHz ^{1}H NMR spectra of bovine AMPA and bovine [Arg^6]AMPA. (A) CIDNP spectrum of bovine AMPA (1.3 mM) with 0.2 mM flavin dye; (B) CIDNP spectrum of bovine [Arg^6]-AMPA (1.1 mM) with 0.2 mM flavin dye; (C) aromatic region of convolution difference spectrum (Campbell et al. 1973) of bovine AMPA; (D) aromatic region of convolution difference spectrum of bovine [Arg^6]AMPA; (E) 360-MHz ^{1}H NMR difference spectrum (aromatic region) of bovine [Arg^6]AMPA minus bovine AMPA. Conditions: 40 °C, pH* 5.0, ionic strength 0.28 M NaCl.

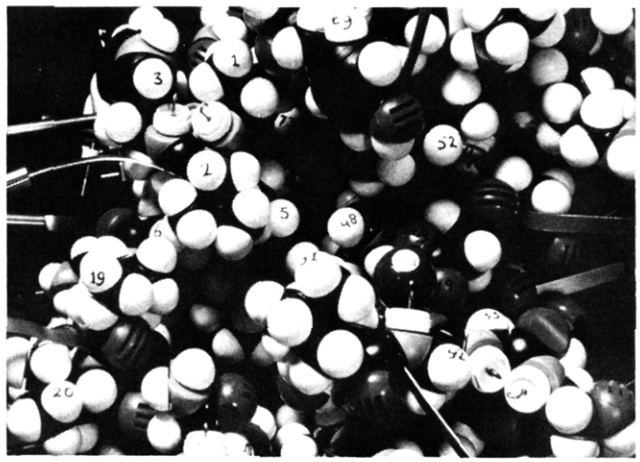

FIGURE 6: Close-up of active site and interface recognition site of CPK model of bovine pancreatic phospholipase A_2 built according to 1.7-Å X-ray structure (Dijkstra et al., 1978; Dijkstra, 1980).

binding of Ca^{2+} ions as well as the inactivation rate with *p*-bromophenacyl bromide of bovine [Arg^6]AMPA is comparable to the corresponding binding of the "native" bovine AMPA and deviate from those of the porcine AMPA, one would be inclined to conclude that the active site of bovine [Arg^6]AMPA is comparable to that of bovine AMPA. However, it has been shown that modification of His^{48} from the active site affects the p*K* of the α-NH^+_3 group of the Ala^1 (Slotboom et al., 1978). Therefore it appears reasonable that changes in the N-terminal region can also influence the active-site construction, thereby improving the enzymatic activity, as found for the Asn^6 substitution by an Arg residue.

Results of the X-ray analysis of bovine PA_2 (Dijkstra, 1980) made clear that the α-NH_2 group of Ala^1 pointing toward the active site (Figure 6) is connected via a H_2O molecule to the active site COO^- of Asp^{99} (Verheij et al., 1980). As can be seen in Figure 6 all residues of the N-terminal sequence Ala^1–Arg^6, which forms a part of an α-helical structure (Ala^1–Lys^{10}), are located close to the active site. For instance the Phe^5 residue, conserved in all phospholipases A_2, is very near the active-site His^{48}. Further substitutions of amino acid residues in this N-terminal sequence, like Phe^5, are therefore in progress to find out their effect on the active-site construction and as a consequence on enzymatic activity.

Acknowledgments

We gratefully acknowledge Mr. R. Dijkman for synthesis and purification of the phospholipids and Dr. G. Donnê-Op den Kelder for her help in building the CPK model of bovine pancreatic phospholipase A_2. We are indebted to Professor Dr. J. Drenth and Dr. B. W. Dijkstra for making available the X-ray coordinates of the bovine pancreatic phospholipase A_2.

Supplementary Material Available

Amino acid analyses of ϵ-amidinated bovine phospholipase A_2 (AMPA), bovine des(Ala^1–Met^8)AMPa, bovine des-(Ala^1–Gly^7)AMPA, bovine [Arg^6]AMPA, and bovine [Asn^6]AMPA as well as of the synthesized peptides Ala-Leu-Trp-Gln-Phe-Arg-Gly and Ala-Leu-Trp-Gln-Phe-Asn-Gly (1 page). Ordering information is given on any current masthead page.

References

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1967) *J. Am. Chem. Soc. 89*, 5012–5017.

Campbell, I. D., Dobson, G. M., & Williams, R. J. P. (1973) *J. Magn. Reson. 11*, 172–181.

Chaiken, I. W., & Komoriya, A. (1978) *Trends Biochem. Sci. 3*, 269–271.

Dam-Mieras, M. C. E. van, Slotboom, A. J., Pieterson, W. A., & Haas, G. H. de (1975) *Biochemistry 14*, 5387–5393.

Deenen, L. L. M. van, and Haas, G. H. de (1964) *Adv. Lipid Res. 2*, 167–234.

Dijkstra, B. W. (1980) Ph.D. Thesis, University of Groningen (The Netherlands).

Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaelen, P. J. (1978) *J. Mol. Biol. 124*, 53–60.

Dutilh, C. E., Doren, P. J. van, Verheul, F. E. A. M., & Haas, G. H. de (1975) *Eur. J. Biochem. 53*, 91–97.

Egmond, M. R., Slotboom, A. J., Haas, G. H. de, Dijkstra, K., & Kaptein, R. (1980) *Biochim. Biophys. Acta 623*, 461–466.

Erickson, B. W., & Merrifield, R. B. (1976) *Proteins, 3rd Ed. 2*, 257–527.

Gisin, B. F. (1973) *Helv. Chim. Acta 56*, 1476–1482.

Gisin, B. F., & Merrifield, R. B. (1972) *J. Am. Chem. Soc. 94*, 3102–3106.

Gray, W. R. (1972) *Methods Enzymol. 25B*, 333–334.

Gross, E., & Witkop, B. (1962) *J. Biol. Chem. 237*, 1856–1860.

Haas, G. H. de, Postema, N. M., Nieuwenhuizen, W., & Deenen, L. L. M. van (1968) *Biochim. Biophys. Acta 159*,

118–129.
Haas, G. H. de, Bonsen, P. P. M., Pieterson, W. A., & Deenen, L. L. M. van (1971) *Biochim. Biophys. Acta 239*, 252–266.
Hruby, V. J., Muscio, F., Groginsky, C. M., Gita, P. M., Saba, D., & Chan, W. Y. (1973) *J. Med. Chem. 16*, 624–629.
Jansen, E. H. J. M., Meijer, H., Haas, G. H. de, & Kaptein, R. (1978) *J. Biol. Chem. 253*, 6346–6347.
Kaptein, R. (1978) in *NMR Spectroscopy in Molecular Biology* (Pullman, B., Ed.) p 211, Reidel, Dordrecht, The Netherlands.
Matsubara, H., & Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun. 35*, 175–181.
Meienhofer, J. (1979) in *The Peptides* (Gross, E., & Meienhofer, J., Eds.) Vol. 1, pp 263–314, Academic Press, London.
Meijer, H. (1979) Ph.D. Thesis, University of Utrecht (The Netherlands).
Meijer, H., Puijk, W. C., Dijkman, R., Foda-van der Hoorn, M. M. E. L., Pattus, F., Slotboom, A. J., & Haas, G. H. de (1979) *Biochemistry 18*, 3589–3597.
Moroder, L., Hallett, A., Wünsch, E., Keller, O., & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651–1653.
Naithani, V. K., Büllesbach, E. E., & Zahn, H. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1363–1366.
Nieuwenhuizen, W., Kunze, H., & Haas, G. H. de (1974) *Methods Enzymol. 32B*, 147–154.
Offord, R. E., & DiBello, C. (1978) *Semisynthetic Peptides and Proteins*, Academic Press, New York and San Francisco.
Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & Haas, G. H. de (1974) *Biochemistry 13*, 1439–1445.
Schroeder, W. A. (1967) *Methods Enzymol. 11*, 351–361.
Slotboom, A. J., & Haas, G. H. de (1975) *Biochemistry 14*, 5394–5399.
Slotboom, A. J., Jansen, E. H. J. M., Pattus, F., & Haas, G. H. de (1978) in *Semisynthetic Peptides and Proteins* (Offord, R. E., & DiBello, C., Eds.) pp 315–349, Academic Press, London.
Soares de Araujo, P., Rosseneu, M. Y., Kremer, J. M. H., Zoelen, E. J. J. van, & Haas, G. H. de (1979) *Biochemistry 18*, 580–586.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237–248.
Verger, R., & Haas, G. H. de (1973) *Chem. Phys. Lipids 10*, 127–136.
Verger, R., Mieras, M. C. E., & Haas, G. H. de (1973) *J. Biol. Chem. 248*, 4023–4034.
Verheij, H. M., Volwerk, J. J., Jansen, E. H. J. M., Puijk, W. C., Dijkstra, B. W., Drenth, J., & Haas, G. H. de (1980) *Biochemistry 19*, 743–750.
Villemoes, P., Christensen, T., & Brunfeldt, K. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 713–719.
Villemoes, P., Christensen, T., & Brunfeldt, K. (1978) *Acta Chem. Scand. B32*, 703–713.
Volwerk, J. J. (1979) Ph.D. Thesis, University of Utrecht (The Netherlands).
Volwerk, J. J., Pieterson, W. A., & Haas, G. H. de (1974) *Biochemistry 13*, 1446–1454.
Volwerk, J. J., Dedieu, A. G. R., Verheij, H. M., Dijkman, R., & Haas, G. H. de (1979) *Recl. Trav. Chim. Pays-Bas 98*, 214–220.
Westall, F. C., Scotchler, J., & Robinson, A. B. (1972) *J. Org. Chem. 37*, 3363–3365.
Wofsy, L., & Singer, S. J. (1963) *Biochemistry 2*, 104–116.
Yamashiro, D., & Li, C. H. (1973) *J. Org. Chem. 38*, 2594–2597.