King, J., & Laemmli, U. K. (1971) *J. Mol. Biol. 62*, 465.
Kohn, L. D., Isersky, C., Zypnik, J., Lenaers, A., Lee, G., & Lapiere, C. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 40.
Lapiere, C. M., Lenaers, A., & Kohn, L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3054.
Lapiere, C. M., Nusgens, B., & Pierard, G. E. (1977) *Connect. Tissue Res. 5*, 21.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335.
Lenaers, A., & Lapiere, C. M. (1976) *Biochim. Biophys. Acta 400*, 121.
Lenaers, A., Ansay, M., Nusgens, B., & Lapiere, C. M. (1971) *Eur. J. Biochem. 23*, 533.
Leung, M. K. K., Fessler, L. I., Greenberg, D. B., & Fessler, J. H. (1979) *J. Biol. Chem. 254*, 224.
Lichtenstein, J. R., Martin, G. R., Kohn, L. D., Byers, P. H., & McKusick, V. A. (1973) *Science (Washington, D.C.) 1982*, 298.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Macarak, E. J., Howard, B. V., & Kefalides, N. A. (1977) *Lab. Invest. 36*, 62.
Martin, G. R., Byers, P. H., & Piez, K. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 42*, 167.
Mayne, R., Vail, M. S., & Miller, E. J. (1978) *Biochemistry 17*, 446.
Miller, E. J. (1976) *Mol. Cell. Biochem. 13*, 165.
Morris, N. P., Fessler, L. I., & Fessler, J. H. (1979) *J. Biol. Chem. 254*, 11024.
Njieha, F. K., Morikawa, T., Tuderman, L., & Prockop, D. J. (1982) *Biochemistry 21*, 757.
Nusgens, B. V., & Lapiere, C. M. (1979) *Anal. Biochem. 95*, 406.
Nusgens, B. V., Goebels, Y., Shinkai, H., & Lapiere, C. M. (1980) *Biochem. J. 191*, 699.
Prockop, D. J., Berg, R. A., Kivirikko, K. I., & Uitto, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G., & Reddi, A. H., Eds.) p 163, Plenum Press, New York.
Ryhänen, L., Tan, E. M. L., Rantala-Ryhänen, S., & Uitto, J. (1982) *Arch. Biochem. Biophys. 215*, 230.
Schofield, J. D., & Prockop, D. J. (1973) *Clin. Orthop. Relat. Res. 97*, 175.
Smith, B. D., Byers, P. H., & Martin, G. R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3260.
Timpl, R., Glanville, R. W., Nowack, H., Wiedemann, H., Fietzek, P. P., & Kühn, K. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1783.
Tryggvason, K., Gehron-Robey, P., & Martin, G. R. (1980) *Biochemistry 19*, 1284.
Tuderman, L., & Prockop, D. J. (1982) *Eur. J. Biochem. 125*, 545.
Tuderman, L., Kivirikko, K. I., & Prockop, D. J. (1978) *Biochemistry 17*, 2948.
Uitto, J., Booth, B. A., & Polak, K. L. (1981) *Biochim. Biophys. Acta 624*, 545.

Different Effects of Substitution of the Near-Invariant Glutamine-4 on the Properties of Porcine and Bovine Pancreatic Phospholipases A_2†

Gustaaf J. M. van Scharrenburg, Wouter C. Puijk, Peter R. Seeger, Gerard H. de Haas, and Arend J. Slotboom*

ABSTRACT: The precise role of the near-invariant Gln-4 residue in bovine and porcine pancreatic phospholipases A_2 was investigated with semisynthesis. Both in bovine and in porcine ϵ-amidinated phospholipases A_2, Gln-4 was substituted by Glu, Asn, and Nle. Binding and kinetic experiments revealed that replacement of Gln-4 by Asn or Nle in bovine phospholipase A_2 eliminates most of the activity, whereas a Gln-4 → Glu substitution affects the enzymatic activity but not the affinity for neutral aggregated substrates. These results clearly indicate the absolute requirement of an $O^{\epsilon 1}$ function of Gln or Glu at the 4-position in *bovine* phospholipase A_2 for a functional lipid binding domain. In contrast, all the *porcine* phospholipase A_2 "mutants" show affinities for micellar aggregates comparable to that of the native enzyme and possess almost full catalytic activity in the kinetic assays with micellar and monomeric short-chain lecithins. The opposite effect of the substitution of Gln-4 by Nle or Asn on the properties of the two enzymes is most likely a result of the presence of a Glu residue at position 71 in the porcine enzyme instead of an Asn as in the bovine enzyme. The porcine phospholipase A_2 is known to possess a second Ca^{2+} binding site located at Glu-71 and affecting the N-terminal region. Recent X-ray data of bovine and porcine phospholipases A_2, showing different conformations of the peptide loop 59–71 in these two enzymes, are in good agreement with this explanation.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of all naturally occurring phospholipids in the presence of Ca^{2+}. The enzyme is found in low concentrations in almost every cell or cellular particle studied (van den Bosch, 1980) but is most abundant in snake venoms and mammalian pancreas (Slotboom et al., 1982). In the pancreas, the enzyme is synthesized as a zymogen. Upon secretion into the gastrointestinal tract, the proenzyme is converted into the active enzyme by limited tryptic proteolysis (de Haas et al., 1968). This active enzyme possesses, in addition to the low activity on monomeric zwitterionic substrates, a 10^3–10^4-fold higher catalytic activity on organized lipid–water interfaces such as occur in micelles (Volwerk & de Haas, 1982).

Modification and substitution of the N-terminal Ala-1 in the porcine pancreatic enzyme revealed that the presence of a free α-NH_3^+ group on Ala-1 is essential for binding to

† From the Laboratory of Biochemistry, State University of Utrecht, University Centre De Uithof, Padualaan 8, 3584 CH Utrecht, The Netherlands. *Received August 25, 1983.* These investigations were carried out under the auspices of The Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.). The key concepts of this paper were presented at the 8th American Peptide Symposium, Tucson, AZ, May 22–27, 1983.

0006-2960/84/0423-1256$01.50/0

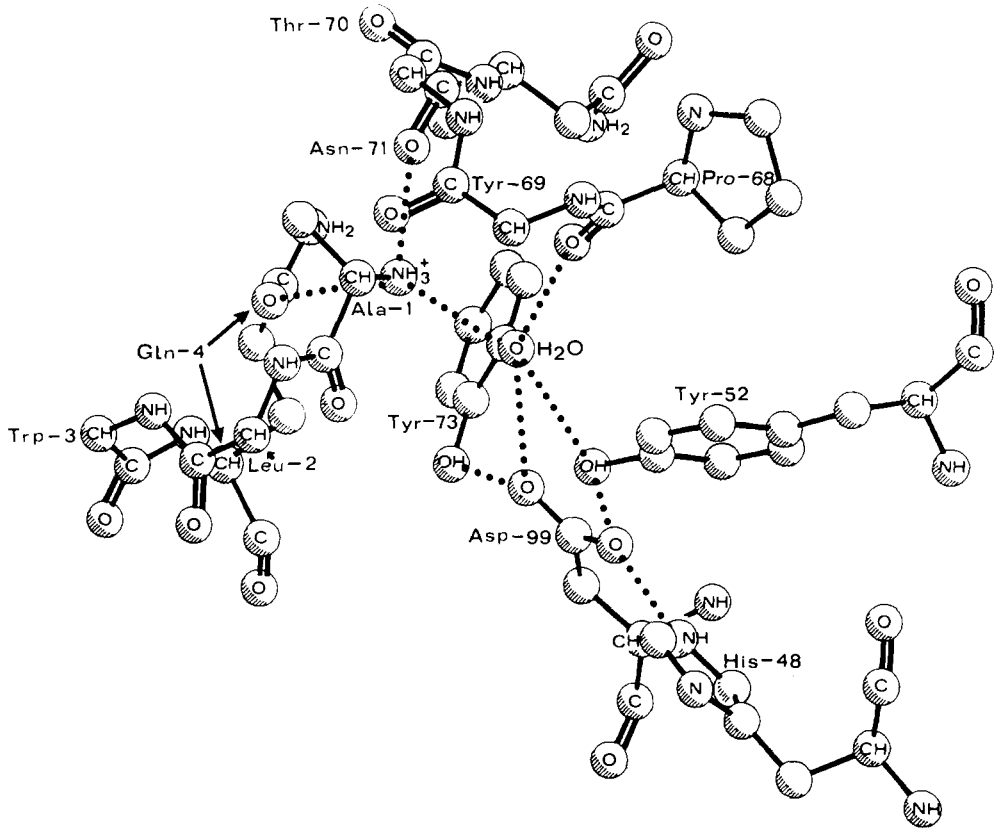

FIGURE 1: Computer drawing of the extended H-bonded system proposed for bovine pancreatic phospholipase A_2 and showing the interactions between the N-terminus, the active site residues, and the amino acid residues between Pro-68 and Asn-71 (according to the 1.7-Å X-ray coordinates; Dijkstra et al., 1981).

neutral lipid–water interfaces and, as a consequence, for enzymatic activity on aggregated substrates (Slotboom & de Haas, 1975). For some snake venom phospholipases A_2, it has recently been reported that the presence of a free α-NH_3^+ group of the N-terminal amino acid residue is a prerequisite for catalytic activity on micellar substrates (Verheij et al., 1981; Meshcheryakova et al., 1982; Aianyan et al., 1982; Magazanik et al., 1979; Randolph & Heinrickson, 1982). In contrast to the pancreatic phospholipases A_2, the absence of a free α-NH_3^+ group of the N-terminal residue in these snake venom phospholipases A_2 does not abolish the affinity for neutral lipid–water interfaces. Apparently, as proposed for pancreatic phospholipase A_2 (Slotboom & de Haas, 1975), an exact juxtaposition of the α-NH_3^+ function and a carboxylate group is also required for full enzymatic activity of snake venom phospholipases A_2. Chemical modification studies (Fleer et al., 1981), as well as X-ray data (Dijkstra et al., 1981, 1983), showed that this carboxylate group belongs to Asp-99, located in the interior of the pancreatic phospholipase A_2. Furthermore, the X-ray analysis showed a direct interaction between the α-NH_3^+ group, the $O^{\epsilon 1}$ atom of Gln-4, the carbonyl oxygen of residue 71, and a fully enclosed H_2O molecule that is in direct contact with Asp-99 and several other active site residues (Figure 1).

The presence of a Gln at position 4 in more than 35 known phospholipase A_2 sequences suggests that this conserved residue, just like the α-NH_3^+ of Ala-1, plays an essential role in the stabilization of an extended hydrogen bridged system and is necessary for the enzyme to display its full catalytic activity. Indeed, substitution of Gln-4 by Nle in bovine pancreatic phospholipase A_2 abolishes completely the catalytic activity of this enzyme toward micellar substrates (van Scharrenburg et al., 1982). Very recently, however, it was found that the phospholipase A_2 from pig ileum (Verger et al., 1982) and the

	1	5	8
BOVINE PANCREATIC	H_3N^+-Ala.Leu.Trp.	Gln.	Phe.Asn.Gly.Met.
PORCINE PANCREATIC	H_3N^+-Ala.Leu.Trp	Gln	Phe.Arg.Ser.Met.
PORCINE INTESTINAL	H_3N^+-Asp.Leu.Leu	Asn	Phe.Arg.Lys.Met.
β_3-BUNGAROTOXIN	H_3N^+-Asn.Leu.Ile.	Asn	Phe.Met.Glu.Met.

FIGURE 2: Comparison of N-terminal amino acid sequences of bovine pancreatic, porcine pancreatic (Slotboom et al., 1982), and porcine intestinal (Verger et al., 1982) phospholipases A_2 and of β-bungarotoxin (Kondo et al., 1982b).

phospholipase A_2 chain of β-bungarotoxin (Kondo et al., 1982a) possess an Asn residue at the 4-position (Figure 2). β-Bungarotoxin does possess considerable phospholipase A_2 activity on micellar lecithins (Kondo et al., 1982a,b), but the intestinal phospholipase A_2 only hydrolyzes monomolecular layers of phosphatidylglycerol (Verger et al., 1982). These data suggest that Gln-4 can be replaced by Asn without a total loss of catalytic activity on lipid–water interfaces.

In order to get more detailed information about the role of Gln-4 in the extended H-bonded system around the α-NH_3^+ group of phospholipase A_2, we substituted Gln-4 by Nle, Glu, and Asn in the bovine enzyme. The same Gln-4 analogues of the porcine pancreatic phospholipase A_2 were also prepared. This enzyme possesses a second, low-affinity Ca^{2+} binding site (Slotboom et al., 1978), which has a large influence on the lipid binding properties (Donné-Op den Kelder et al., 1981) and on the pK_a of the α-amino group, the trigger of the H-bonded system (Slotboom et al., 1978). The present paper describes the preparation of the analogues and compares the effects of Gln-4 substitutions on the catalytic and lipid binding properties of the bovine and porcine pancreatic phospholipases A_2.

Materials and Methods

Most of the materials used and methods applied have been

described in detail previously (Slotboom & de Haas, 1975; van Scharrenburg et al., 1981, 1982). N^{α}-Fmoc[1] amino acids were purchased from Chemalog, Chemical Dynamics Corp.

Amino acid analyses were performed by the method of Spackman et al. (1958) on a Kontron liquid chromatograph Liquimat III amino acid analyzer. Protein concentrations were calculated from the absorbance at 280 nm with an $E_{1cm}(1\%)$ of 12.5 for all the ϵ-amidinated phospholipases A_2. N-Terminal amino acid residues were determined by dansylation according to the method of Gray (1972).

Measurements of Enzymatic Activities. 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 a 1.5-fold higher sodium deoxycholate concentration were used. Kinetic measurements, with micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine and monomeric [2,3-bis(hexanoylthio)propyl]phosphocholine, were performed as described before [see de Haas et al. (1971) and Volwerk et al. (1979), respectively].

UV Absorption Difference Spectroscopy. UV absorption difference spectra were recorded on an Aminco DW-2-a spectrophotometer equipped with a Midan data analyzer coupled to an Apple II desk-top computer. Measurements were done at 25 °C, and binding parameters were obtained as described by Hille et al. (1981). Experimental conditions were 50 mM sodium acetate, 0.1 M sodium chloride, and 0.1 M $CaCl_2$ at pH 6.00.

Fluorescence spectroscopy was done as described by van Dam-Mieras et al. (1975).

Monolayer Experiments. The surface barostat method was performed with a zero-order trough and 1,2-didecanoyl-*sn*-glycero-3-phosphocholine and 1,2-didodecanoyl-*sn*-glycero-3-phospho-*rac*-glycerol to measure substrate hydrolysis and induction time at pH 6.00 as described by Verger (1980).

Synthesis of Peptides. *N-t*-Boc-Gly or *N-t*-Boc-Ala was esterified to chloromethylated polystyrene resin that has been 1% cross-linked with divinylbenzene (1.34 mmol of Cl/g of resin) according to the method of Gisin (1973). The heptapeptides H_2N-Ala-Leu-Trp(For)-Gln-Phe-Arg-Ala (I), H_2N-Ala-Leu-Trp(For)-Asn-Phe-Arg-Ala (II), H_2N-Ala-Leu-Trp(For)-Nle-Phe-Arg-Ala (III), and H_2N-Ala-Leu-Trp-(For)-Asn-Phe-Asn-Gly (IV) were prepared by solid-phase synthesis (Erickson & Merrifield, 1976), with N^{α}-*t*-Boc-amino acids and starting with *N-t*-Boc-Gly or *N-t*-Boc-Ala resin (1.1 mmol of Ala or Gly/g of resin). The computerized peptide synthesizer (SYN 1), developed by the Danish Institute of Protein Chemistry (Villemoes et al., 1978), used the same automatic protocol for coupling and deprotection as described by van Scharrenburg et al. (1981).

The peptides H_2N-Ala-Leu-Trp-Glu(OBu^t)-Phe-Arg-Ala (V) and H_2N-Ala-Leu-Trp-Glu(OBu^t)-Phe-Asn-Gly (VI) were also synthesized by the solid-phase method, with the following strategy: the two C-terminal amino acids were coupled as their dicyclohexylcarbodiimide-activated N^{α}-*t*-Boc derivatives to the Gly or Ala resin by the above-mentioned procedure. The four N-terminal amino acids (starting with Glu-OBu^t) were successively coupled as the symmetrical anhydrides of the N^{α}-Fmoc-protected amino acids, prepared in situ according to the procedure of Meienhofer et al. (1979). A complete cycle using the symmetrical N^{α}-Fmoc-amino acid anhydrides consisted of the following steps: (1) CH_2Cl_2, 3 times for 3 min; (2) 50% piperidine/CH_2Cl_2 for 5 min; (3) 50% piperidine/CH_2Cl_2 for 30 min; (4) CH_2Cl_2, 2 times for 3 min; (5) DMF, 2 times for 3 min; (6) H_2O/dioxane (1:2 v/v), 2 times for 5 min; (7) DMF, 3 times for 3 min; (8) CH_2Cl_2, 3 times for 3 min; (9) 3 equiv of in situ prepared Fmoc-amino acid anhydride in DMF/CH_2Cl_2 (1:3 v/v) for 30 min and, after 15 min, 1 equiv of 0.5 M DIEA in CH_2Cl_2 added; (10) CH_2Cl_2, 3 times for 3 min; (11) DMF, 3 times for 3 min; (12) EtOH, 3 times for 3 min.

After the last complete cycle, in which N^{α}-Fmoc-Ala was coupled to the peptide resin, the Fmoc group was removed by performing steps 1–8. During the synthesis of peptides I–III, the δ-guanidino group of arginine was protected by the tosyl moiety. In the case of peptide V, the arginine was protected by a nitro group. The γ-carboxyl groups of glutamic acid in peptides V and VI were esterified with a *tert*-butyl group, and the indole nitrogen of tryptophan in peptides I–IV was protected by the formyl moiety.

Peptides I–IV were cleaved from the solid support by treatment with liquid HF in the presence of 10% anisole for 30 min at 0 °C. Cleavage of peptides V and VI from the resin was done by catalytic transfer hydrogenation, with palladium acetate and ammonium formate as described by Anwer & Spatola (1981). During this cleavage, the nitro group used for Arg protection in peptide V is removed simultaneously.

Enzymatic degradation of peptides V and VI by leucine aminopeptidase (Light, 1967) followed by amino acid analysis revealed that the *tert*-butyl protection of the γ-carboxyl group of the glutamic acid was still present after the hydrogenation procedure.

After cleavage, the peptides were extracted from the resin with acetic acid, acetic acid/H_2O (1:1 v/v), or trifluoroacetic acid. Extracts were combined, diluted with cold water, and lyophilized. The yield of crude peptides was 80% for peptides I–IV and 40–50% for the Glu(OBu^t)-containing peptides V and VI, on the basis of the amount of starting *N-t*-Boc-Gly or *N-t*-Boc-Ala resin.

Purification of Peptides. All the crude peptides were purified in 50-mg portions by high-performance LC on a preparative C_{18} reversed-phase Polygosil column (25 cm × 22.7 mm; particle size 10 μm; Altex) with a linear gradient (2 times 400 mL) of 10–90% of acetonitrile in H_2O containing 0.1% TFA at a flow rate of 15 mL/min. The α-amino groups of the purified peptides were reacted with di-*tert*-butyl dicarbonate according to the procedure of Moroder et al. (1976). Subsequently, a second purification was done by high-performance LC on a C_{18} reversed-phase Polygosil column as described above. The homogeneity of the N^{α}-*t*-Boc peptides was checked by analytical high-performance LC (van Scharrenburg et al., 1981). Amino acid analysis of the pure N^{α}-*t*-Boc heptapeptides showed for all six peptides the correct amino acid composition.

Preparation of Porcine Des-(Ala^1–Ser^7)-AMPA. Porcine AMPA was split selectively at Arg-6 by treatment with 10% TPCK-treated trypsin (w/w). Subsequently, the new N-terminal Ser-7 was removed by a preparative Edman degradation, yielding porcine des-(Ala^1–Ser^7)-AMPA (Slotboom & de Haas, 1975).

[1] Abbreviations: Fmoc, (9-fluorenylmethoxy)carbonyl; *t*-Boc, *tert*-butyloxycarbonyl; For, formyl; OBu^t, *O-tert*-butyl; DMF, dimethylformamide; DIEA, *N,N*-diisopropylethylamine; EtOH, ethanol; LC, liquid chromatography; TFA, trifluoroacetic acid; AMPA, ϵ-amidinated phospholipase A_2; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; tosyl, *p*-toluenesulfonyl; NO_2, nitro; CM, carboxymethyl; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance; CIDNP, chemically induced dynamic nuclear polarization; IEP, isoelectric point. Other abbreviations used follow the IUPAC–IUB Commission on Biochemical Nomenclature Recommendations (1967a,b, 1972).

Preparation of Bovine Des-(Ala[1]–Gly[7])-AMPA. The preparation of this protein fragment is described by van Scharrenburg et al. (1981).

Preparation of the Semisynthetic Bovine and Porcine AMPA Analogues. Coupling of the pure N^{α}-*t*-Boc heptapeptides (I–VI) to the desired protein fragment, bovine des-(Ala[1]–Gly[7])-AMPA or porcine des-(Ala[1]–Ser[7])-AMPA, was performed by using the mixed carbonic anhydride method (Meienhofer, 1979; van Scharrenburg et al., 1981). The resulting *N*-*t*-Boc-[Trp[3](For)]AMPA analogues were desalted on a Sephadex G-25 coarse column (3 × 80 cm) equilibrated with 1% acetic acid, and the fractions containing the protein peaks were pooled and lyophilized. The crude *N*-*t*-Boc-[Trp[3](For)]AMPAs were then deformylated by treatment with 1 M NH_4HCO_3 (pH 9.00) for 24 h (Yamashiro & Li, 1973). After deformylation, the semisynthetic *N*-*t*-Boc-AMPA analogues 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 6.00 or 5.00 as described previously (van Scharrenburg et al., 1981). Fractions containing the *N*-*t*-Boc-AMPA analogues were pooled, dialyzed and lyophilized, and treated with 2 mL of anhydrous trifluoroacetic acid under N_2 at room temperature. After 15–30 min, the mixture was diluted with a large excess of cold H_2O and lyophilized immediately. The semisynthetic bovine and porcine AMPA analogues were obtained in 15–30% yield after chromatography on DEAE-cellulose (van Scharrenburg et al., 1981, 1982). All the semisynthetic proteins were homogeneous on gel electrophoresis (pH 9.3) (Studier, 1973), and dansylation showed Ala as the N-terminal amino acid residue in all cases.

Results

Preparation of Bovine and Porcine AMPA Analogues. The semisynthetic approach to obtain phospholipase A_2 "mutants" requires the protection of the ϵ-NH_2 groups of all lysyl residues to avoid undesired side reactions during coupling or degradation steps. ϵ-Amidinated bovine and porcine pancreatic prophospholipases A_2 were used to prepare the N-terminally substituted enzyme analogues. The ϵ-amidinated phospholipases A_2 (AMPAs) behave nearly identical with the corresponding native enzymes with respect to catalytic activity and substrate binding properties (Slotboom & de Haas, 1975; van Scharrenburg et al., 1981).

Bovine des-(Ala[1]–Met[8])-AMPA was obtained by cleavage of the ϵ-amidinated prophospholipase A_2 with CNBr at the unique Met-8 residue (Figure 2). Subsequent coupling of the *N*-hydroxysuccinimide ester of *N*-*t*-Boc-L-Met followed by treatment with TFA yielded bovine des-(Ala[1]–Gly[7])-AMPA, the protein entity used for condensation with the synthetic peptides.

For the preparation of the N-terminally shortened *porcine* AMPA, CNBr cleavage at Met-8 cannot be used because of the presence of an additional methionine residue at position 20 in this enzyme. Trypsin was, therefore, used to selectively split the Arg[6]–Ser[7] linkage in porcine AMPA, followed by a preparative Edman degradation furnishing the desired porcine des-(Ala[1]–Ser[7])-AMPA (van Scharrenburg et al., 1983).

The N-terminal heptapeptides (I–IV) containing Asn or Nle at position 4 were synthesized by the solid-phase procedure with N^{α}-*t*-Boc-protected amino acids and were removed from the resin by liquid HF (van Scharrenburg et al., 1981). During solid-phase synthesis of the Glu-4 containing heptapeptides (V and VI), N^{α}-*t*-Boc protection was also used for the coupling of the three C-terminal amino acid residues. The four N-terminal amino acid residues were attached by using symmetrical N^{α}-Fmoc-protected amino acid anhydrides. The use of basic conditions to split off the N^{α}-Fmoc has the advantage that the *tert*-butyl group used to protect the γ-carboxyl function of the Glu-4 is not removed during the remainder of the synthesis. The side-chain protection of Glu-4 must be maintained for the unambiguous coupling of the heptapeptides V and VI to the α-NH_2 group of the N-terminally shortened bovine and porcine AMPA fragments, and therefore, liquid HF cannot be used to cleave peptides V and VI from the resin. Removal of these peptides from the resin with retention of the side-chain protection of Glu-4 was mediated by hydrogenation as described recently by Anwer & Spatola (1981). Although these authors reported a recovery of more than 90% of their peptide from the resin by this procedure, we succeeded after two successive treatments to obtain only 50% yield with our peptides. We protected Arg-6 in peptide V with a nitro group, which is removed simultaneously during the cleavage of the peptide from the resin by hydrogenation.

Arg-6 in peptide V cannot be protected with the tosyl moiety, because it is not removed during the hydrogenation procedure used for cleavage of the peptide from the resin. To check the presence of Glu(OBu[t]) in peptides V and VI, as well as the absence of NO_2-Arg in peptide V, both purified peptides were digested with leucine aminopeptidase. Quantitative amino acid analyses of the reaction mixtures showed in both peptides the presence of 1 mol of Glu(OBu[t]) in addition to 1 mol of each of the other constituting amino acid residues. No nitroarginine was found to be present in the reaction mixtures of peptide V.

After protection of the α-NH_2 group with di-*tert*-butyl dicarbonate, the N^{α}-*t*-Boc heptapeptides thus obtained were covalently coupled to the respective bovine des-(Ala[1]–Gly[7])- and porcine des-(Ala[1]–Ser[7])-AMPA fragments by using the mixed-anhydride method. Subsequent purification by ion-exchange chromatography before and after deprotection gave the pure AMPA analogues in a yield of 15–30%. The feasibility of the procedures applied has been established previously by preparing semisynthetically the original bovine and porcine AMPAs, which were found to be indistinguishable from the respective "native" AMPAs (van Scharrenburg et al., 1981, 1983).

The heptapeptides coupled to the bovine des-(Ala[1]–Gly[7])-AMPA fragment possess only one substitution at the 4-position as compared to the original sequence. The heptapeptides coupled to the porcine des-(Ala[1]–Ser[7])-AMPA possess in addition to the substitutions at the 4-position another substitution at the C-terminal position, that is, Ser-7 → Ala. This latter substitution was introduced for practical reasons, because yields for coupling peptides that contain Ser as the C-terminal residue are lower as compared to peptides with Ala as the C-terminal residue (van Scharrenburg et al., 1983). For this reason, all the 4-substituted porcine AMPAs possess an Ala instead of a Ser at position 7. The effect of the Ser-7 → Ala substitution on the properties of porcine AMPA has been described recently (van Scharrenburg et al., 1983). As a result, it was found that about 70% of the catalytic activity on micellar substrates was retained, while the affinity for micellar substrate analogues remained unchanged following the Ser-7 → Ala substitution in porcine AMPA (see also Table II).

Kinetic and Substrate Binding Properties of Bovine AMPA Analogues. As can be seen from Table I, substitution of Gln-4 by Asn in bovine AMPA reduces catalytic activity both on micellar and monomeric substrates. Only residual activities in the order of 10% of native AMPA could be measured. These results are comparable with the data reported previously

Table I: Comparison of Kinetic and Binding Properties of Semisynthetic Bovine ε-Amidinated [Glu⁴]-, [Asn⁴]-, and [Nle⁴]Phospholipases A_2 with Native ε-Amidinated Bovine Phospholipase A_2

AMPA analogue	sp act. (egg yolk assay) (μequiv min^{-1} mg^{-1})[a]	V_{max} (micellar L-dioctanoyllecithin assay) (μequiv min^{-1} mg^{-1})[b]	k_{cat}/K_M[c] [monomeric bis(thiohexanoyl)-lecithin assay] (s^{-1} M^{-1})[c]	K_D (binding of micellar *cis*-9-octadecenylphosphocholine) (mM)[d]
native AMPA (Gln-4)	50 ± 3	2300 ± 160	172 ± 10	5.0 ± 0.6
Glu-4	7.5 ± 0.8	1050 ± 50	98 ± 4	4.6 ± 0.3
Asn-4	4.5 ± 0.6	158 ± 20	18 ± 2	*f*
Nle-4	*e*	70 ± 20	45 ± 5	*f*

[a] Determined at pH 8 as described previously (van Scharrenburg et al., 1982). [b] Determined by titration of fatty acid liberated with 10.3 mM NaOH at pH 6 and 45 °C (50 mM $CaCl_2$, 10 mM acetate, and 0.1 M NaCl) as described previously (de Haas et al., 1971; van Scharrenburg et al., 1982). [c] Determined at pH 8.5 and 25 °C as described by Volwerk et al. (1979). [d] Determined at pH 6.0 (50 mM acetate, 0.1 M NaCl, and 0.1 M $CaCl_2$) by ultraviolet difference spectroscopy (Hille et al., 1981; van Scharrenburg et al., 1982). [e] No detectable activity. [f] No detectable binding.

Table II: Comparison of Kinetic and Binding Properties of Semisynthetic Porcine ε-Amidinated [Glu⁴, Ala⁷]-, [Asn⁴, Ala⁷]-, and [Nle⁴,Ala⁷]Phospholipases A_2 with ε-Amidinated Porcine [Ala⁷]Phospholipase A_2 and Native ε-Amidinated Porcine Phospholipase A_2[a]

AMPA analogue	sp act. (egg yolk assay) (μequiv min^{-1} mg^{-1})	V_{max} (micellar L-dioctanoyllecithin assay) (μequiv min^{-1} mg^{-1})	k_{cat}/K_M [monomeric bis(thiohexanoyl)-lecithin assay] (s^{-1} M^{-1})	K_D (binding of micellar *cis*-9-octadecenylphosphocholine) (mM)
native AMPA (Gln-4, Ser-7)	310 ± 15	2220 ± 100	476 ± 30	0.16 ± 0.02
Gln-4, Ala-7	240 ± 10	1340 ± 50	440 ± 25	0.09 ± 0.01
Glu-4, Ala-7	125 ± 10	910 ± 50	425 ± 25	0.09 ± 0.01
Asn-4, Ala-7	85 ± 5	950 ± 50	920 ± 90	0.33 ± 0.02
Nle-4, Ala-7	6 ± 1	864 ± 50	132 ± 17	0.13 ± 0.03

[a] See footnotes *a–d* of Table I.

for the bovine [Nle⁴]AMPA. This mutant possesses a slightly higher activity in the monomeric bis(thiohexanoyl)lecithin assay than does [Asn⁴]AMPA (van Scharrenburg et al., 1982). The low activities of the bovine [Nle⁴]- and [Asn⁴]AMPA analogues toward micellar substrates are most likely due to the fact that these AMPA analogues have lost their affinities for neutral substrates. As can be seen from Table I, these two bovine AMPA analogues do not bind to micelles of the substrate analogue *cis*-9-octadecenylphosphocholine. This loss of a functional lipid binding domain is not found upon replacement of Gln-4 by Glu in bovine AMPA. The K_D value for the binding of bovine [Glu⁴]AMPA to the micellar substrate analogue is almost identical with that of native bovine AMPA. The [Glu⁴]AMPA possesses about 50% of the activity of native AMPA by the micellar L-dioctanoyllecithin assay, which also demonstrates that the [Glu⁴]analogue recognizes and binds to neutral lipid–water interfaces. Bovine [Glu⁴]-AMPA possesses only 15% of the specific activity of native AMPA at pH 8 in the egg yolk assay (Table I). A plausible explanation for this remarkably low value could be the simultaneous presence of the negative charge on Glu-4 in the N-terminal lipid binding domain at pH 8 and of the negatively charged mixed micelles of egg yolk lecithin and sodium deoxycholate.

pH Dependency of Fluorescence Intensity of Bovine AMPA Analogues. Figure 3, part II, shows the effect of pH on the fluorescence intensity of Trp-3 in native AMPA and semisynthetic bovine [Asn⁴]-, [Nle⁴]-, and [Gln⁴]AMPAs. The catalytically active [Glu⁴]AMPA has a high fluorescence intensity as in native bovine AMPA. In contrast, the bovine [Nle⁴]- and [Asn⁴]AMPA analogues, both devoid of catalytic activity and affinity for neutral lipid–water interfaces, possess a much lower fluorescence intensity. The lower fluorescence intensity of the latter two AMPA analogues must be due to a different, more quenching environment surrounding Trp-3 in these two AMPA analogues compared to that in the native

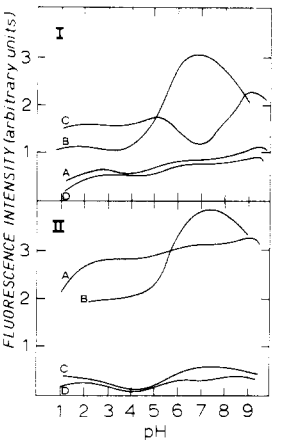

FIGURE 3: pH dependence of fluorescence intensities of porcine (I) and bovine (II) AMPA analogues: (I) (A) [Gln⁴, Ala⁷]AMPA; (B) [Glu⁴, Ala⁷]AMPA; (C) [Asn⁴, Ala⁷]AMPA; (D) [Nle⁴, Ala⁷]-AMPA. (II) (A) [Gln⁴]AMPA (=native); (B) [Glu⁴]AMPA; (C) [Asn⁴]AMPA; (D) [Nle⁴]AMPA. Experimental conditions: 1 mM Tris, 1 mM sodium acetate, 0.1 M NaCl, 14.2 μM protein. Excitation was at 295 nm, and intensities were measured at the emission maximum (342 nm) at 25 °C.

AMPA and [Glu⁴]AMPA. The fluorescence intensity of [Glu⁴]AMPA (Figure 3II-B) shows a 2-fold increase in going from pH 4 to 7.5. Since the only difference with the native bovine AMPA is the Gln-4 → Glu substitution, this rise of the fluorescence signal could be attributed to the deprotonation of the Glu-4 residue adjacent to the tryptophan at position 3. The high pK_a value of 5.8 for this Glu-4 is probably a consequence of the rather hydrophobic microenvironment created by the neighboring Trp-3 and Phe-5 residues.

Kinetic and Substrate Binding Properties of Porcine AMPA Analogues. The data in Table II show that for porcine AMPA, substitution of the near-invariant Gln-4 by Glu, Asn, or Nle does not eliminate the enzymatic activity toward mi-

cellar substrates nor the binding to micelles of *cis*-9-octadecenylphosphocholine. As compared to [Ala^7]AMPA, the Gln-4 → Glu substitution has almost no effect on the properties of the enzyme. Both in the micellar L-dioctanoyllecithin and in the monomeric bis(thiohexanoyl)lecithin assays, the activities are greater than 65% of that found with [Ala^7]AMPA. Only the specific activity, determined with negatively charged mixed micelles in the egg yolk assay, is lower but not as low as that found for the same substitution in the bovine enzyme. The affinity of [Glu^4, Ala^7]AMPA for the micellar substrate analogue *cis*-9-octadecenylphosphocholine at pH 6 is identical with that of native AMPA, while the affinity of [Asn^4, Ala^7]AMPA is about 4 times lower. This decrease in the affinity toward aggregated substrates can be attributed to a perturbation of the hydrogen-bonded system around the N-terminal Ala-1, but a functional lipid binding domain is still present. Although the Gln-4 → Asn substitution causes a considerable decrease in the specific activity when assayed in the egg yolk assay, the V_{max} value obtained in the micellar L-dioctanoyllecithin assay is less affected. The high activity of porcine [Asn^4, Ala^7]AMPA in the monomeric bis(thiohexanoyl)lecithin assay at pH 8.5, viz., more than 200% of that of [Ala^7]AMPA, is remarkable. A similarly high activity in the monomeric assay has been found for enzymes with a blocked or modified Ala-1 such as prophospholipase A_2 or transaminated phospholipase A_2. It is also surprising that the substitution Gln-4 → Nle is not lethal for the porcine AMPA. The affinity of [Nle^4, Ala^7]AMPA for micellar *cis*-9-octadecenylphosphocholine is identical with that of native AMPA, and the V_{max} value in the micellar short-chain lecithin assay is 64% of that of [Ala^7]AMPA. The Gln-4 → Nle substitution, however, dramatically decreases the activity on monomeric substrates and in particular the specific activity in the egg yolk assay.

pH Dependency of Fluorescence Intensity of Porcine AMPA Analogues. The effect of pH on the fluorescence intensity of the Trp-3 in the porcine analogues is shown in Figure 3I. A remarkable increase in fluorescence intensity is evident on increasing the pH from 4 to 7.5 with the porcine [Glu^4, Ala^7]AMPA. Glu-4 in this enzyme has approximately the same apparent pK_a value as that found for bovine [Glu^4]-AMPA. The fluorescence intensity of Trp-3 in [Glu^4, Ala^7]- and [Asn^4, Ala^7]AMPAs is more than 2-fold increased as compared to that of [Ala^7]AMPA, while [Nle^4, Ala^7]AMPA possesses a fluorescence intensity comparable to that of [Ala^7]AMPA. These results thus suggest that Trp-3 in the catalytically active porcine [Asn^4, Ala^7]- and [Nle^4, Ala^7]-AMPAs is not in a low-quenching environment.

Discussion

The presence of Asn or Nle at position 4 in bovine pancreatic phospholipase A_2 abolishes completely the affinity for micelles composed of the substrate analogue *cis*-9-octadecenylphosphocholine (Table I). This lack of affinity for micelles explains the very low catalytic activity of these two "mutants" on the micellar substrate L-dioctanoyllecithin. The findings obtained with bovine [Asn^4]- and [Nle^4]AMPA show unambiguously the need for an O$^{\epsilon 1}$ of glutamine or glutamic acid for the maintenance of a functional lipid binding domain. The 1.7-Å X-ray structure (Dijkstra et al., 1981; Dijkstra, 1980) of bovine phospholipase A_2 suggested an important role of the conserved Gln-4 in stabilizing the H-bonded system around the N-terminus that is essential for a lipid binding domain (Figure 1). The interaction between the α-NH_3^+ and the O$^{\epsilon 1}$ or Gln-4 or Glu-4 seems critical for an exact internal juxtaposition of this α-amino group of bovine phospholipase A_2. The lack of affinity of bovine [Asn^4]AMPA toward neutral lipid–water interfaces demonstrates that even a small shift in the position of the amide carbonyl oxygen in the side chain of Gln-4 disrupts the functional conformation of the lipid binding site. The disruption of H-bridge connections around the α-NH_3^+ group influences the conformation of the α-helical N-terminal region (residues 1–13) that contains amino acids like Leu-2, Trp-3, and Asn-6 important for the hydrophobic interaction with micelles. The conformational change in this N-terminal region is reflected in the decreased fluorescence intensity of Trp-3 in both bovine [Nle^4]- and [Asn^4]AMPA compared to native bovine and bovine [Glu^4]-AMPA (Figure 3I). Previous results obtained with bovine [Nle^4]AMPA, from the photo CIDNP ^{1}H NMR technique and an immunological assay, also indicated that a drastic conformational change occurs following substitution of Nle for Gln at position 4, giving rise to a more inward position of Trp-3 (van Scharrenburg et al., 1982). Substitution of Gln-4 by Asn or Nle has, as one would expect, the same consequence as blocking or modifying the α-NH_3^+ function of Ala-1, that is, the loss of a functional lipid binding domain. The hydrogen-bonding system (Figure 1) also links the α-NH_3^+ group, via a H_2O molecule to the active site couple His-48-Asp-99. Due to the substitution of Gln-4 by Asn or Nle, the α-NH_3^+ function and the catalytic center are probably no longer in close contact.

The absolute requirement for an O$^{\epsilon 1}$ at position 4 in *bovine* phospholipase A_2 contrasts remarkably with findings obtained with the same mutants of the *porcine* pancreatic enzyme. Substitution of Gln-4 by Nle or Asn in porcine phospholipase A_2 does not give proteins devoid of affinity and enzymatic activity toward neutral aggregated substrates (Table II). Obviously, Gln-4 is of minor importance in porcine pancreatic phospholipase A_2. The opposite effect of the substitution of Gln-4 by Asn or Nle in the bovine and porcine phospholipases A_2 is most likely due to differences in the H-bridge system around the α-NH_3^+ function of these enzymes. Differences in the microenvironment of the N-terminal amino groups present in bovine and porcine phospholipase A_2 are also reflected in their pK_a values. The pK_a values of the α-NH_3^+ functions in the native porcine and bovine enzymes are 8.4 and 8.9, respectively, as determined by ^{13}C NMR titrations (Slotboom et al., 1978; Jansen et al., 1979). Furthermore, Slotboom et al. (1978) showed that, at basic pH values, a second Ca^{2+} ion was bound with low affinity to porcine phospholipase A_2. Binding of this second Ca^{2+} ion perturbs Trp-3 and shifts the pK_a of the α-NH_3^+ group from 8.4, in the absence of Ca^{2+}, to 9.4 at 250 mM Ca^{2+}. The authors concluded that porcine pancreatic phospholipase A_2 possessed a second low-affinity Ca^{2+} binding site close to the N-terminal part of the protein.

A similar shift in the pK_a of the α-NH_3^+ group or perturbation of Trp-3 is not observed upon addition of high amounts of Ca^{2+} to bovine phospholipase A_2. The second, low-affinity Ca^{2+} binding site was very recently located on Glu-71 of both the porcine and equine phospholipases A_2 (Donné-Op den Kelder et al., 1983) but not in the bovine enzyme (Asn-71). The bovine enzyme possesses only the catalytic Ca^{2+} binding site. The Glu-71 of the porcine phospholipase A_2 is a carboxylate having an abnormal pK_a of 6.25. Deprotonation of this residue results in a complete loss of affinity for neutral lipid–water interfaces (Donné-Op den Kelder et al., 1981). Binding of Ca^{2+} restores micellar binding and shifts, as mentioned above, the pK_a of the α-NH_3^+ group to 9.4. This is 0.5 pH unit higher than the pK_a of the α-NH_3^+ function in bovine phospholipase A_2 (pK_a = 8.9). As a matter of fact,

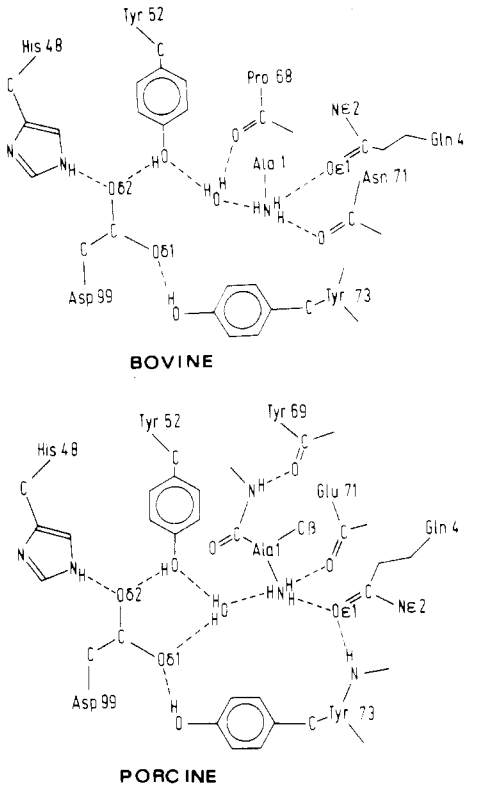

FIGURE 4: Comparison of the schematically represented H-bonded systems of bovine and porcine pancreatic phospholipases A_2 (Dijkstra et al., 1983).

the presence of an Asn at position 71 in the bovine phospholipase and a Glu at the same position in porcine phospholipase A_2 is the only striking dissimilarity between the residues involved in the H-bridge system around the α-NH_3^+ group of these phospholipases A_2 (Figure 4). The other amino acid residues involved are conserved in all the pancreatic phospholipases A_2. Therefore, the differences in pK_a values of the α-NH_3^+ functions and the opposite effects of a substitution of Gln-4 by Asn or Nle in the bovine and porcine enzymes could be attributed to the differing residues at position 71.

The X-ray structure of porcine pancreatic phospholipase A_2, recently refined to 2.6 Å (Dijkstra et al., 1983), shows a great resemblance to the X-ray structure of bovine pancreatic phospholipase A_2 (1.7 Å) (Dijkstra et al., 1981). According to the authors, one of the main differences in the backbone conformation of these two enzymes is the structure of the peptide loop 59–71. A short α-helical structure in bovine phospholipase A_2, occurring between residues 59 and 66, is not present in the porcine enzyme. The residues 67–71 in the porcine phospholipase A_2 form a short stretch of 3_{10} helix, whereas in the bovine protein this part is a random coil. These structural differences are thought to be a result of the only two substitutions in the peptide loop 59–71, that is, Val-63 → Phe-63 and Asn-71 → Glu-71 in the bovine and porcine enzymes, respectively. The different backbone structures in this region also have consequences for the interactions around the α-NH_3^+ functions of these enzymes. Figure 4 shows schematically the interactions between the N-terminus and the active site for both the bovine and porcine pancreatic phospholipases A_2. It is clear that the conserved active site residues His-48, Asp-99, Tyr-52, and Tyr-73 occupy exactly the same positions around the α-NH_3^+ group in both enzymes. These pancreatic enzymes differ, as expected, in the interaction of the $O^{\epsilon 1}$ of Gln-4 and in the interaction of the peptide bond carbonyl functions of the loop 67–71 with the N-terminus. These relatively small variations in the structure of the H-bonded systems must account for the deviation in the pK_a values of the α-NH_3^+ group and the opposite effect of the substitution of Gln-4 by Asn or Nle in the porcine and bovine enzymes.

Verger et al. (1982) purified recently a phospholipase A_2 from pig ileum with an Asn at position 4 and an activity directed only toward phosphatidylglycerol monolayers. It does not seem likely that the Asn at position 4 of the intestinal phospholipase A_2 is entirely responsible for this substrate specificity. Monolayer experiments with porcine [Nle^4, Ala^7]- and [Asn^4, Ala^7]AMPA using lecithin and phosphatidylglycerol did not indicate any change in substrate specificity. Furthermore, the phospholipase A_2 chain of β-bungarotoxin (Kondo et al., 1982a,b) has been reported to possess an Asn at position 4, and it also possesses phospholipase A_2 activity in the egg yolk assay (Kondo et al., 1982a,b), as well as in the micellar L-dioctanoyllecithin assay (H. M. Verheij, personal communication). Obviously, the presence of Asn at the 4-position in the porcine intestinal phospholipase A_2, β-bungarotoxin, and porcine pancreatic phospholipase A_2 does not eliminate enzymatic activity of these three enzymes. The near-invariant character of Gln-4 in over 35 sequences of phospholipase A_2 suggests, however, that in most cases the interaction between Gln-4 and the α-amino group is essential for full enzymatic activity.

Acknowledgments

We gratefully acknowledge R. Dijkman for synthesis and purification of the phospholipids. We are indebted to Dr. A. L. Speck (Department of Structural Chemistry, University of Utrecht) for his help in the preparation of Figure 1. We thank Dr. B. W. Dijkstra (State University of Groningen) for providing Figure 4. We are much indebted to Dr. P. Child (University of Toronto, Canada) for carefully proofreading the manuscript.

Registry No. I, 88780-54-5; II, 88285-99-8; III, 88780-55-6; IV, 88780-56-7; V, 88801-68-7; VI, 88801-69-8; Gln, 56-85-9; Asn, 70-47-3; Nle, 327-57-1; Glu, 56-86-0; phospholipase A_2, 9001-84-7; L-dioctanoyllecithin, 36012-02-9; *cis*-9-octadecenylphosphocholine, 76622-80-5; bis(thiohexanoyl)lecithin, 76622-79-2.

References

Aianyan, A. E., Surina, E. I., Miroshnikov, A. I., Gevod, V. S., & Ksenzhek, O. S. (1982) *Bioorg. Khim. 8*, 364–376.

Anwer, M. K., & Spatola, A. F. (1981) *Tetrahedron Lett. 22*, 4369–4372.

de Haas, G. H., Postema, N. M., Nieuwenhuizen, W., & van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta 159*, 118–129.

de Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., & van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta 239*, 252–266.

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

Dijkstra, B. W., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1981) *J. Mol. Biol. 147*, 97–123.

Dijkstra, B. W., Renetseder, R., Kalk, K. H., & Drenth, J. (1983) *J. Mol. Biol. 168*, 163–179.

Donné-Op den Kelder, G. M., Hille, J. D. R., Dijkman, R., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4074–4078.

Donné-Op den Kelder, G. M., de Haas, G. H., & Egmond, M. R. (1983) *Biochemistry 22*, 2470–2478.

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

Fleer, E. A. M., Verheij, H. M., & de Haas, G. H. (1981) *Eur. J. Biochem. 113*, 283–288.
Gisin, B. F. (1973) *Helv. Chim. Acta 56*, 1476–1482.
Gray, W. R. (1972) *Methods Enzymol. 25B*, 333–334.
Hille, J. D. R., Donnê-Op den Kelder, G. M., Sauve, P., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4068–4073.
IUPAC–IUB Commission on Biochemical Nomenclature Recommendations (1967a) *Biochemistry 6*, 362–364.
IUPAC–IUB Commission on Biochemical Nomenclature Recommendations (1967b) *Biochemistry 6*, 3287–3292.
IUPAC–IUB Commission on Biochemical Nomenclature Recommendations (1972) *Biochemistry 11*, 1726–1732.
Jansen, E. H. J. M., van Scharrenburg, G. J. M., Slotboom, A. J., de Haas, G. H., & Kaptein, R. (1979) *J. Am. Chem. Soc. 101*, 7393–7399.
Kondo, K., Toda, H., Narita, K., & Lee, C. (1982a) *J. Biochem. (Tokyo) 91*, 1519–1530.
Kondo, K., Toda, H., Narita, K., & Lee, C. (1982b) *J. Biochem. (Tokyo) 91*, 1531–1548.
Light, A. (1967) *Methods Enzymol. 11*, 426–436.
Magazanik, L. G., Gotgilf, I. M., Slavnova, T. I., Miroshnikov, A. I., & Apsalon, U. R. (1979) *Toxicon 17*, 477–488.
Meienhofer, J. (1979) *Peptides (N.Y.) 1*, 263–314.
Meienhofer, J., Waki, M., Heimer, E. P., Lambros, T. J., Makofshe, R. C., & Chang, C. D. (1979) *Int. J. Pept. Protein Res. 13*, 35–42.
Meshcheryakova, E. A., Aianyan, A. E., Kostetskii, P. V., & Miroshnikov, A. I. (1982) *Bioorg. Khim. 8*, 349–363.
Moroder, L., Hallett, A., Wünsch, E., Keller, O., & Wersin, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1651–1653.
Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32B*, 147–154.
Randolph, A., & Heinrickson, R. L. (1982) *J. Biol. Chem. 257*, 2155–2161.
Slotboom, A. J., & de Haas, G. H. (1975) *Biochemistry 14*, 5394–5399.
Slotboom, A. J., Jansen, E. H. J. M., Vlijm, H., Pattus, F., Soares de Araujo, P., & de Haas, G. H. (1978) *Biochemistry 17*, 4593–4600.
Slotboom, A. J., Verheij, H. M., & de Haas, G. H. (1982) *Phospholipids: New Biochemistry* (Hawthorne, J. N., & Ansell, G. B., Eds.) Vol. 4, pp 359–434, Elsevier, Amsterdam.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237–248.
van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5393.
van den Bosch, H. (1980) *Biochim. Biophys. Acta 604*, 191–246.
van Scharrenburg, G. J. M., Puijk, W. C., Egmond, M. R., de Haas, G. H., & Slotboom, A. J. (1981) *Biochemistry 20*, 1584–1591.
van Scharrenburg, G. J. M., Puijk, W. C., Egmond, M. R., van der Schaft, P. H., de Haas, G. H., & Slotboom, A. J. (1982) *Biochemistry 21*, 1345–1352.
van Scharrenburg, G. J. M., Puijk, W. C., de Haas, G. H., & Slotboom, A. J. (1983) *Eur. J. Biochem. 133*, 83–89.
Verger, R. (1980) *Methods Enzymol. 64*, 340–392.
Verger, R., Ferrato, F., Mansbach, C. M., & Pieroni, G. (1982) *Biochemistry 21*, 6883–6889.
Verheij, H. M., Egmond, M. R., & de Haas, G. H. (1981) *Biochemistry 20*, 94–99.
Villemoes, D., Christensen, T., & Brunfeldt, K. (1978) *Acta Chem. Scand., Ser. B B32*, 703–713.
Volwerk, J. J., & de Haas, G. H. (1982) *Lipid-Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol 1, pp 69–149, Wiley, New York.
Volwerk, J. J., Dedieu, A. G. R., Verheij, H. M., Dijkman, R., & de Haas, G. H. (1979) *Recl. Trav. Chim. Pays-Bas 98*, 214–220.
Yamashiro, D., & Li, C. H. (1973) *J. Org. Chem. 38*, 2594–2597.