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Specific Transformations at the N-Terminal Region of Phospholipase A_2†

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ABSTRACT: Treatment of porcine pancreatic prophospholipase A_2 with methyl acetimidate converted all lysine residues into ϵ-acetimidolysine residues. Enzymatically active ϵ-amidinated phospholipase A_2 (AMPA) was obtained from the ϵ-amidinated zymogen by limited tryptic proteolysis cleaving the Arg_7–Ala_8 bond. AMPA was used to prepare des-Ala^8-, des-(Ala^8,Leu^9)- and des-(Ala^8,Leu^9,Trp^{10})-AMPA by successive Edman degradations, and des-(Ala^8–Arg^{13})-AMPA by selective splitting of the Arg_{13}–Ser_{14} bond by trypsin. Structural analogues of AMPA with different N-terminal amino acid residues, viz., D-Ala, β-Ala, and Gly, have been prepared by reacting des-Ala^8-AMPA with the corresponding *N*-*t*-Boc-*N*-hydroxysuccinimide esters of these amino acids. Similarly, the only Trp_{10} residue has been substituted for Phe by coupling of des-(Ala^8,-Leu^9,Trp^{10})-AMPA with *N*-*t*-Boc-L-Ala-L-Leu-L-Phe-*N*-hydroxysuccinimide ester. The feasibility of these substitutions has been proven unambiguously by the retroconversion of des-Ala^8-AMPA and of [Ala^7]AMPA into AMPA having identical enzymatic activity as the starting AMPA. The single Trp_{10} residue in native phospholipase A_2 and its zymogen was specifically sulfenylated using *o*-nitrophenylsulfenyl chloride. The homogeneous proteins were kinetically analyzed using short-chain lecithins in the monomeric and micellar region. All modified AMPA analogues, except those in which two or more of the N-terminal amino acid residues are removed, show enzymatic activities toward monomeric substrate comparable to that of AMPA, indicating that the active site region is still intact. Only [Gly^8]-, [β-Ala^8]-, and [Ala^8,Leu^9,Phe^{10}]AMPA exhibit a dramatic increase in enzymatic activity similar to that of AMPA upon passing the critical micellar concentration (cmc) of the substrate. From these results it can be concluded that the N-terminal region of the enzyme requires a very precise architecture in order to interact with lipid–water interfaces and consequently to display its full enzymatic activity.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of fatty acid ester bonds at the 2 position of 3-*sn*-phosphoglycerides (van Deenen and de Haas, 1964). Although porcine pancreatic phospholipase A_2 has some activity toward substrates present in monomeric solutions there is a tremendous increase in enzymatic activity when substrate is present as an organized lipid–water interface (de Haas et al., 1971). In contrast, however, prophospholipase A_2, although possessing about 50% of the activity of the enzyme toward monomeric substrate, does not show the increase in enzymatic activity when the substrate concentration passes the critical micellar concentration. This fundamental difference between the zymogen and the active enzyme has been postulated to be due to the presence of an interface recognition site (IRS)[1] in the enzyme which is not present in the zymogen (Verger et al., 1973; Pieterson et al., 1974). This site is supposed to give a specific interaction with certain organized lipid–water interfaces, followed by a conformational change in the enzyme with concomitant optimization of the active site architecture. Furthermore it has been demonstrated that the formation of the IRS is controlled by the protonation of the α-amino group of the N-terminal Ala_8 (Figure 1) having a p*K* value close to 8.1 (Pieterson et al., 1974) and which presumably forms an ion pair with a buried carboxylate group (Abita et al., 1972). In agreement with earlier observations that the IRS is not only functionally but also topographically distinct from the active site (Pieterson et al., 1974), the results of spectroscopic studies and protection against tryptic attack provide strong evidence that the hydrophobic N-terminal part of the enzyme, Ala_8-Leu-Trp-Gln-Phe-Arg_{13} (Figure 1), is directly involved in the IRS (van Dam-Mieras et al., 1975).

In order to delineate further the role of the amino acids involved in the IRS, N-terminally modified and substituted enzyme analogues are required. The main purpose of the present paper is to describe convenient methods to prepare such analogues by various chemical and enzymatic procedures (Figure 2).

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[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature recommendations (*Biochemistry 6,* 362 (1967); *Biochemistry 6,* 3287 (1967); *Biochemistry 11,* 1726 (1972)) were used throughout. Other abbreviations used are: *t*-Boc, *tert*-butyloxycarbonyl; Ptc, phenylthiocarbamoyl; NPS, *o*-nitrophenylsulfenyl; Dip-F, diisopropyl phosphorofluoridate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IRS, interface recognition site; AMPA, ϵ-amidinated phospholipase A_2; cmc, critical micellar concentration.

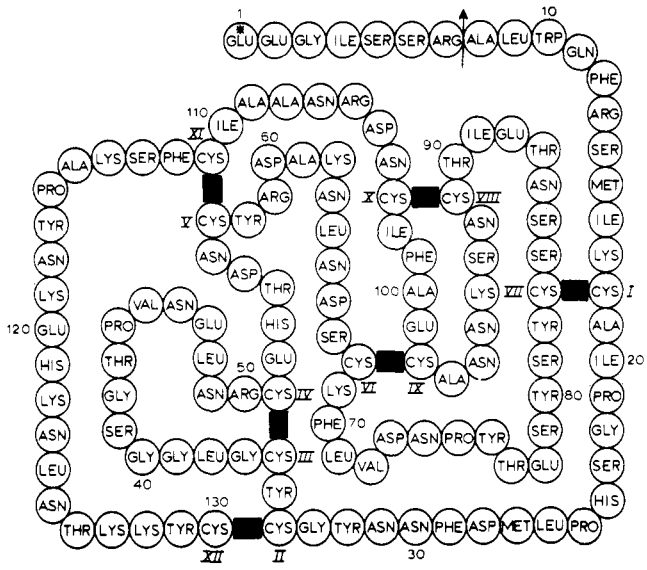

FIGURE 1: Primary structure of porcine pancreatic prophospholipase A_2. * stands for pyroglutamic acid.

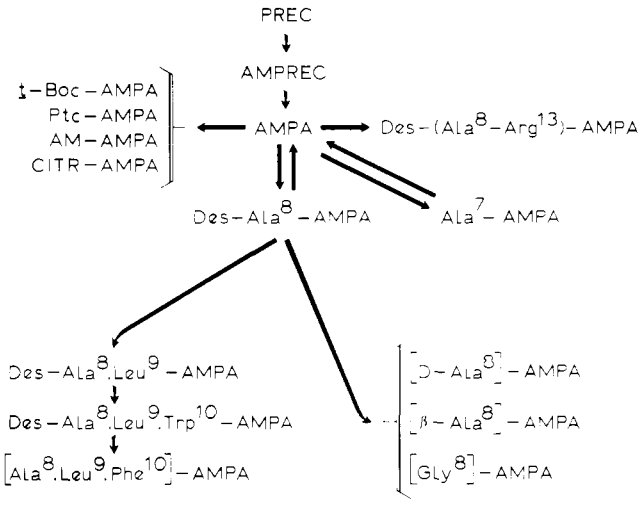

FIGURE 2: Scheme depicting chemical and enzymatic transformations at the N-terminal region of ε-amidinated phospholipase A_2 (AMPA); PREC refers to prophospholipase A_2, AMPREC refers to ε-amidinated PREC; *t*-Boc-, Ptc-, AM-, and CITR-AMPA refer to α-*N*-*tert*-butyloxycarbonyl-, α-phenylthiocarbamoyl-, α-amidinated, and α-citraconylated AMPA, respectively.

Experimental Section

Materials

N-*t*-Boc-*N*-hydroxysuccinimide esters of L-Ala, Gly, and β-Ala were obtained from Fluka A.G. (Switzerland) and Fox Chemical Co. D-Ala and *o*-nitrophenylsulfenyl chloride were purchased from Fluka A.G. (Switzerland), L-Leu-L-Phe was from Schwarz/Mann, and β-Ala was from B.D.H. Ltd. (England). Chemical and stereochemical purity of the various amino acid derivatives were checked by established procedures (thin-layer chromatography on silicic acid and optical rotation) and found to be in accordance with reported values. Methyl acetimidate hydrochloride was prepared according to the procedure of Hunter and 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. 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). Trypsin and TPCK-treated trypsin were products of Serva (Germany). Carboxypeptidases A and B (Dip-F-treated suspensions) were products of Worthington Biochem. Corp. Dip-F was purchased from EGA Chemie KG (Germany). All the other chemicals used were of the highest purity available.

Methods

Optical rotations were measured in a Lichtelektrisches Präzisions polarimeter 0.005° (Carl Zeiss, Jena, Germany). Melting points were determined on a Kofler hot plate and are uncorrected. Element analyses were carried out under the supervision of Mr. W. J. Buis at the Micro-Analytical Department of the Institute of Organic Chemistry TNO, Utrecht, The Netherlands. Protein concentrations for phospholipase A_2 and its zymogen were calculated from the absorbances at 280 nm with an $E_{1\,cm}(1\%)$ of 13.0 and 12.3, respectively, unless stated otherwise. 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. Samples were hydrolyzed for 24 hr at 110° in evacuated, sealed tubes with 6 *N* HCl. Trp was determined similarly by addition of 4% of thioglycollic acid as described by Matsubara and Sasaki (1969). β-Ala eluted at 1.13 times the retention time of Phe from the long column and a ninhydrin value of 0.28 relative to Phe was determined. Starch gel electrophoresis at pH 5.5 (0.05 *M* sodium acetate) as described by de Haas et al. (1968) was used to check the purity of the various proteins. High voltage electrophoresis and chromatography on paper were performed as described previously (de Haas et al., 1970). Carboxyl terminal analyses were done as described previously (de Haas et al., 1970). Amino terminal analyses were carried out by the Edman procedure as modified by Peanasky et al. (1969) and by the dansylation procedure of Gray and Hartley (1963) as modified by Gros and Labouesse (1969). Oxidation of disulfide bridges was performed as described by Hirs (1956) using performic acid. Enzyme activities were determined using the titrimetric assay procedure with egg-yolk lipoproteins as substrates (Nieuwenhuizen et al., 1974) and with monomeric and micellar short-chain lecithins (de Haas et al., 1971).

Sulfenylation of native phospholipase A_2 and its zymogen was performed essentially according to the procedure of Scoffone et al. (1968). To a solution of 70 mg (5 μmol) of protein dissolved in 7 ml of 20% HOAc was added 70 mg of solid *o*-nitrophenylsulfenyl chloride (375 μmol) in five equal portions over a total period of 90 min. The reaction mixture was shaken vigorously in the dark at room temperature. At the end of the reaction the mixture was centrifuged from excess reagent, and the supernatant was dialyzed for 24 hr at 4° and lyophilized. Pure *o*-nitrophenylsulfenylphospholipase A_2 and *o*-nitrophenylsulfenyl prophospholipase A_2 were obtained by chromatography on CM-cellulose as described below for the preparation of AMPA.

N-*t*-Boc-D-Ala was prepared from D-Ala following the procedure described by Schnabel (1967) for L-Ala. *N*-*t*-Boc-D-Ala had mp 83–84° and $[\alpha]^{25}D$ +21.5° (acetic acid, 0.1783 g/ml) (cf. Schnabel (1967): mp 83–84°; $[\alpha]D$ −25.2° and −22.4° for *N*-*t*-Boc-L-Ala). *Anal.* Calcd for $C_8H_{15}NO_4$ (mol wt 189.2): C, 50.78; H, 7.99; N, 7.39.

Found: C, 50.7; H, 8.1; N, 7.5. Conversion of *N-t*-Boc-D-Ala into its *N*-hydroxysuccinimide ester was performed essentially according to the method outlined by Anderson et al. (1964). The *N-t*-Boc-D-Ala-*N*-hydroxysuccinimide ester had mp 166–167° and $[\alpha]^{25}D$ +54.5° (dioxane, 0.0396 g/ml) (cf. Anderson et al. (1964): mp 143–144° and 167° for another form; and $[\alpha]D$ −49° for *N-t*-Boc-L-Ala-*N*-hydroxysuccinimide ester). *Anal.* Calcd for $C_{12}H_{18}N_2O_6$ (mol wt 286.26): C, 50.34; H, 6.34; N, 9.78. Found: C, 50.3; H, 6.3; N, 9.5. The purity of *N-t*-Boc-D-Ala and its *N*-hydroxysuccinimide ester was checked by thin-layer chromatography on SiO_2 using chloroform–methanol–acetic acid (85:15:5 v/v); detection by spraying with ninhydrin followed by heating.

Conversion of L-Leu-L-Phe into the *N-t*-Boc-L-Ala-L-Leu-L-Phe-*N*-hydroxysuccinimide ester was effected as described by Yaron et al. (1974) by reaction of the dipeptide L-Leu-L-Phe with *N-t*-Boc-L-Ala-*N*-hydroxysuccinimide ester. Subsequently the carboxylate group was activated according to the procedure outlined by Anderson et al. (1964). The N-protected tripeptide was shown to be homogeneous on high-voltage paper electrophoresis and upon amino acid analysis equimolar amounts of Ala, Leu, and Phe were found.

Preparation of N-Terminally Modified AMPA Analogues

Amidination of Prophospholipase A_2. The zymogen (5.5 g) was dissolved in 800 ml of distilled H_2O and the pH of the solution adjusted to 9.2 with 5 *N* NaOH. To the magnetically stirred solution 125 g of freshly prepared methyl acetimidate hydrochloride was added over a period of 2 hr at room temperature keeping the pH of the solution between 9.1 and 9.3 by the addition of 5 *N* NaOH. When all methyl acetimidate hydrochloride was added, the solution was kept for another 2 hr at room temperature while stirring and keeping the pH of the solution between 9.1 and 9.3 by the addition of 4 *N* HCl. Excess reagents were then removed by exhaustive dialysis at 4° for 48 hr. After lyophilization 5.5 g of crude ε-amidinated prophospholipase A_2 were obtained which were purified at 4° by chromatography on a DEAE-cellulose column (5 × 70 cm) equilibrated with 5×10^{-3} *M* Tris (pH 7.3) and developed with 5 l. of a linear salt gradient reaching 0.1 *M* NaCl in the same buffer. Fractions containing ε-amidinated zymogen were pooled, dialyzed at 4° for 24 hr, and lyophilized, yielding 3.75 g (68%) of electrophoretically homogeneous ε-amidinated prophospholipase A_2. During dialysis and lyophilization of ε-amidinated zymogen 0.1 *M* Dip-F (2% v/v) in absolute 2-propanol was added to inhibit traces of proteolytic enzymes. Reaction of the ε-amidinated zymogen with 2,4-dinitrofluorobenzene followed by 6 *N* HCl hydrolysis for 18 hr 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[2] of ε-amidinated prophospholipase A_2 was found to be identical with that of the native protein except for the modified lysine residues.

ε-Amidinated phospholipase A_2 (AMPA) was obtained by limited tryptic proteolysis of ε-amidinated prophospholipase A_2. Therefore a magnetically stirred solution of 3.5 g of the ε-amidinated zymogen in 5 m*M* $CaCl_2$ solution (300 ml) adjusted to pH 7.0 was incubated at 0° with 0.06% (w/w) of trypsin. The activation was followed by measuring the increase in enzymatic activity using the egg-yolk assay (Nieuwenhuizen et al., 1974). At maximal activation the tryptic action was stopped by the addition of pure Dip-F and the sample chromatographed on CM-cellulose equilibrated at pH 6.0. Fractions containing AMPA having a constant specific activity over the protein peak were pooled, dialyzed for 24 hr at 4°, and lyophilized yielding 2.5 g (75%) of AMPA, which was found to be pure on gel electrophoresis. The amino acid composition of AMPA was found to be identical with that of native phospholipase A_2. End group analysis using the dansyl procedure showed the presence of only Ala.

Peptide chain shortening at the N-terminus was carried out by the Edman procedure as modified by Peanasky et al. (1969). AMPA (0.5 g) was dissolved in 45 ml of distilled H_2O and the pH adjusted to 9.0 with *N*-ethylmorpholine. To this solution were added an equal volume of Edman buffer (absolute ethanol–*N*-ethylmorpholine, 500:60 v/v, pH 8.6) followed by 6 ml of phenyl isothiocyanate. The mixture was shaken vigorously at 40° under N_2 for 1 hr. After extraction of excess phenyl isothiocyanate with benzene (four times), the aqueous phase was lyophilized and chromatographed at 4° on a CM-cellulose column (2 × 35 cm) equilibrated with 5×10^{-3} *M* sodium acetate (pH 6.0) and developed with 1 l. of a linear salt gradient reaching 0.3 *M* sodium chloride in the same buffer. Fractions containing phenylthiocarbamoyl-AMPA (Ptc-AMPA) were pooled, dialyzed for 24 hr at 4°, and lyophilized. The material obtained was treated with 5 ml of anhydrous trifluoroacetic acid for 15 min at 40° under N_2 and the mixture was lyophilized immediately after dilution with cold water. Purification of the des-Ala^8-AMPA was performed on CM-cellulose using similar conditions as described above for the Ptc-AMPA. Fractions containing the pure des-Ala^8-AMPA were cwmbined, dialyzed at 4° for 24 hr, and lyophilized. End group analysis of des-Ala^8-AMPA using the dansyl procedure showed Leu to be the only N-terminal amino acid.

In the same way starting from des-Ala^8-AMPA two consecutive Edman cycles, removing Leu_9 and Trp_{10}, furnished des-(Ala^8,Leu^9)-AMPA and des-(Ala^8,Leu^9,Trp^{10})-AMPA.

The average yield for one complete Edman cycle including two times chromatography on CM-cellulose was found to be 80%. Routinely the purity of the intermediary Ptc-protein as well as of the shortened protein was checked by gel electrophoresis. The amino acid analyses of the N-terminally degraded AMPA analogues agreed within the accuracy with the expected values.

Peptide Chain Elongation at the N-Terminus. To 0.2 g of des-Ala^8-AMPA dissolved in 40 ml of a 0.1 *M* Hepes buffer (pH 8.0) a solution of 4 ml of dimethylformamide containing a tenfold molar excess of *N-t*-Boc-L-Ala-*N*-hydroxysuccinimide ester was added. After 3–4 hr at room temperature the reaction mixture was dialyzed at 4° for 24 hr and lyophilized. The material obtained was treated for 20 min at room temperature with 40 ml of a 1 *M* H_2NOH solution at pH 7.0.[3] After dialysis for 24 hr at 4° and lyoph-

[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 for examination to the reviewers.

[3] Amino acid analysis of the product revealed the incorporation of more than one alanine residue/protein molecule, presumably due to ester formation. The hydroxylamine treatment as described above resulted in the quantitative removal of all but one residue of alanine/protein molecule, without effecting other modifications in the protein molecule.

ilization the *N-t*-Boc-AMPA was purified by chromatography on CM-cellulose at pH 6.0 in the same way as described above for peptide chain shortening at the N-terminus. Fractions containing pure *N-t*-Boc-AMPA were pooled, dialyzed for 24 hr at 4°, and lyophilized. Finally, the *t*-Boc group was removed by treatment with 2 ml of anhydrous trifluoroacetic acid at room temperature under N_2 for 15 min. A large excess of cold distilled H_2O was then added and the sample lyophilized as soon as possible. Purification of the regenerated AMPA was performed by chromatography on CM-cellulose as described above for peptide chain shortening at the N-terminus. Fractions containing the pure AMPA were pooled, dialyzed at 4° for 24 hr, and lyophilized.

Similarly, des-Ala^8-AMPA was used to prepare [D-Ala^8]-, [β-Ala^8]-, and [Gly^8]AMPA, while AMPA itself was converted to [Ala^7]AMPA.[4]

Substitution of the single Trp residue in AMPA by Phe was effected in the same way by chain elongation of des-(Ala^8,Leu^9,Trp^{10})-AMPA with *N-t*-Boc-L-Ala-L-Leu-L-Phe-*N*-hydroxysuccinimide ester.

The yield of coupling of one amino acid residue including deblocking and purifications was found to be 70% except for β-Ala and the tripeptide Ala-Leu-Phe. In these latter cases reproducible lower yields (30%) were observed and large amounts of unreacted protein were recovered. All proteins prepared by chain elongation were found to be homogeneous on gel electrophoresis and to have a correct amino acid composition.

Blocking of the α-Amino Group in AMPA. Amidination of the α-amino group of AMPA was effected by to successive reactions with methyl acetimidate as described above for the preparation of ϵ-amidinated prophospholipase A_2. The completely amidinated protein was purified on CM-cellulose in the same way as AMPA.

Blocking of the α-amino function with the *t*-Boc group was performed by reaction of AMPA (60 mg) with *t*-Boc-azide (120 μl) dissolved in 6 ml of a mixture of dimethylformamide and water (3:1 v/v) in the presence of 6 mg of sodium bicarbonate according to the procedure of Geiger and Langner (1973). After 6 hr at 35° the reaction mixture was desalted by passage through a Sephadex G-25 fine column (pH 9.0) and the protein fraction was further purified by chromatography on CM-cellulose as described above for AMPA. Electrophoretically pure *t*-Boc-AMPA was obtained in 90% yield, which after removal of the *t*-Boc group by trifluoroacetic acid treatment regenerated AMPA having the same specific activity as the starting AMPA.

Tryptic Cleavage of Arg_{13}–Ser_{14} in AMPA. Incubation of AMPA with TPCK-treated trypsin gives rise to a pseudo-first-order reaction with loss of enzymatic activity. The fact that in AMPA the lysine residues are no longer susceptible to trypsin attack suggests that the loss of activity is correlated with a trypsin splitting of one of the Arg–X linkages. In our previous sequence studies (de Haas et al., 1970) it was found that trypsin cleavage at Arg_{50} and Arg_{59} is a rather slow process and therefore it might be anticipated that the above loss of AMPA activity is caused by splitting either at the Arg_{13} or Arg_{106} level. In order to determine which peptide linkage(s) are split by trypsin, a large scale incubation was done: 0.2 g of AMPA dissolved in 200 ml of 0.01 *M* Tris (pH 8.0)–10^{-2} *M* $CaCl_2$ was magnetically stirred at room temperature. To the solution was added 20 mg of TPCK-treated trypsin, dissolved in 1 ml of the same buffer. The decrease in enzymatic activity was followed using the egg-yolk assay and the tryptic action was stopped by addition of Dip-F after 3 hr when approximately 30% of the enzymatic activity was left. After lyophilization the reaction mixture was fractionated over Sephadex G-25 in 10^{-3} *M* HCl yielding a protein and a peptide containing fraction. Upon amino acid analysis the latter was shown to be the N-terminal hexapeptide Ala^8-Leu-Trp-Gln-Phe-Arg^{13} (cf. Figure 1). Subsequent purification of the protein fraction was effected by chromatography on CM-cellulose as described for the preparation of AMPA. Des-(Ala^8–Arg^{13})-AMPA was obtained in a yield of 66% while unreacted AMPA, possessing the same specific activity as AMPA, was recovered in a yield of 25%. Des-(Ala^8–Arg^{13})-AMPA gave only one spot on gel electrophoresis and its amino acid composition was in good agreement with that to be expected after removal of the N-terminal hexapeptide. Analysis of the terminal amino acids by the Edman and dansylation technique showed the presence of only Ser (and its degradation products), whereas no amino acids were detectable after carboxypeptidase A or B attack. The possibility that trypsin had induced additional splittings in the polypeptide chain with the formation of new terminal amino acids not accessible to the reagents could not be excluded. Also after performic acid oxidation of the disulfide bridges only Ser was found as the N-terminal amino acid and no amino acids were released upon carboxypeptidase A or B attack. Moreover, elution of the oxidized des-(Ala^8–Arg^{13})-AMPA over Sephadex G-50 fine showed only one protein peak having an amino acid composition identical with that of des-(Ala^8–Arg^{13})-AMPA before oxidation. In conclusion, controlled trypsin hydrolysis of AMPA gives rise to a selective cleavage of the peptide bond Arg_{13}–Ser_{14}.

Results and Discussion

An attractive approach to explore the role in enzyme function of essential amino acids located at the N-terminal region of enzymes is their selective substitution. In their elegant studies describing the latent reactivity of trypsinogen, Robinson et al. (1973) demonstrated the feasibility of this approach by chemically shortening as well as by elongating the N-terminus of ϵ-guanidated trypsin with one amino acid residue. At the same time Lode et al. (1973, 1974b) successfully substituted the N-terminal as well as the adjacent amino acid residue of *Clostridium acidi-urici* ferredoxin, a protein which does not contain lysine residues. Similar substitutions in *Clostridium M-E* ferredoxin containing lysine residues resulted in the incorporation of considerably more than one residue, presumably caused by incomplete protection of ϵ-amino groups with *t*-Boc-azide (Lode et al., 1974a). Recently, Garner and Gurd (1975) prepared an amino terminal [1-^{13}C]-adduct to sperm whale myoglobin. Their preparation, however, was heavily contaminated due mainly to unwanted blocking of the α-amino group during protection of the ϵ-amino groups with methyl acetimidate as well as to incomplete deblocking.

Specific substitution of amino acid residues at the N-terminal region of enzymes by removing these residues using the Edman procedure and subsequent replacement by reaction with activated *N*-protected amino acid derivatives requires that all ϵ-aminolysine residues are selectively pro-

[4] [Ala^7]AMPA was subjected to one Edman cycle yielding AMPA as described under peptide chain shortening at the N-terminus.

tected without blocking the α-amino group.[5] In the present study this was easily achieved by limited tryptic proteolysis of fully ϵ-amidinated prophospholipase A_2 which gives rise to ϵ-amidinated phospholipase A_2 (AMPA) having a free α-amino group. AMPA possesses about 60% of the activity of the native enzyme: V_{max} values are 1120 and 1920 μequiv min^{-1} mg of protein^{-1}, respectively, while both enzymes have the same pH optimum (6.0) and a similar affinity for Ca^{2+}, K_{Ca} = 0.5 and 0.4 mM, respectively.[6] It is therefore not necessary to deblock the ϵ-acetimidolysine residues to study the effect of amino acid substitutions on enzymatic activity. In order to correlate possible differences in the properties of AMPA analogues due to amino acid substitutions at the N-terminal region it is a prerequisite to prove the feasibility of the applied procedures. Therefore, as shown in Figure 2 des-Ala^8-AMPA was reconverted into AMPA by coupling with *N*-*t*-Boc-L-Ala-*N*-hydroxysuccinimide ester. In addition [Ala^7]AMPA was transformed into AMPA by Edman degradation. AMPA obtained from either pathway was found to have identical enzymatic activity as the starting AMPA (Table I). Furthermore no differences could be detected between the "semisynthetic" AMPA's and the starting AMPA with respect to amino acid composition and behavior on gel electrophoresis.[7] These results therefore allow the selective substitution of the N-terminal L-Ala residue in AMPA by other amino acid residues, e.g., Gly, β-Ala, and D-Ala as indicated in Figure 2.

In principle it is possible to remove more amino acid residues from the N-terminus by consecutive Edman cycles as demonstrated by the preparation of des-(Ala^8,Leu^9)- and des-(Ala^8,Leu^9,Trp^{10})-AMPA which were obtained in overall yields of 64 and 51%, respectively. The latter protein was used successfully to selectively substitute the only Trp for Phe by coupling of the protein with the activated N-protected tripeptide Ala-Leu-Phe (Figure 2).

Mainly because of the required purifications there is, however, a rapidly diminishing yield and consequently a limitation to the stepwise removal of N-terminal amino acids on a preparative scale by the Edman procedure. In order to extend the possibility to substitute amino acid residues located further from the N-terminal amino acid, it is therefore advantageous to selectively split peptide fragments from the N-terminal site. Obviously the success of such an approach is dependent on the preferential susceptibility to enzymatic or chemical cleavage of a single, conveniently located peptide bond. Especially specific cleavage by proteolytic enzymes is attractive because it is a very mild procedure, although in particular cases also CNBr splitting can be used.

From the above results it is evident that trypsin specifically splits the Arg$_{13}$–Ser$_{14}$ linkage releasing the N-terminal hexapeptide. It has been demonstrated that the remaining protein des-(Ala^8–Arg^{13})-AMPA, obtained in 66% yield, consists of one single polypeptide chain without any splittings. So far noncovalent recombination of the hexapeptide and des-(Ala^8–Arg^{13})-AMPA did not lead to restoration of enzymatic activity and probably the presence of a covalent bond is essential for phospholipase A_2 activity in contrast to the well-known enzymatically active ribonuclease S-peptide–S-protein system.

Table I: Comparison of Kinetic Parameters[a] and of $E_{1\,cm}$ (1%) Values at 280 nm of ϵ-Amidinated Phospholipase A_2 (AMPA) and Various N-Terminally Modified AMPA Analogues.

	Monomeric L-Dihexanoyllecithin		Micellar L-Dioctanoyllecithin	$E_{1\,cm}$ (1%) at 280 nm
	K_m (mM)	V_{max}[b]	V_{max}[b]	
AMPA	4.2	8.5	1120	13.0
[Gly^8] AMPA	1.2	4.3	910	13.0
[β-Ala^8] AMPA	3.5	2.3	303	13.0
[Ala^8,Leu^9,Phe^{10}] AMPA	4.2	4.3	430	10.1
AMPA[c]	Not determined		1180	13.0
AMPREC	20–25	4.0	3	12.3
[Ala^7] AMPA		1.7	8	13.0
Des-[Ala^8] AMPA		0.7	7	13.0
[D-Ala^8] AMPA		2.0	32[d]	13.0
AM-AMPA		1.8	6	13.0
Ptc-AMPA		6.7	7	14.0
t-Boc-AMPA		5.0	6	13.0
Des-[Ala^8–Arg^{13})-AMPA	No detectable activity			8.8

[a] K_m values measured on organized lipid–water interfaces are not given because these have no physical meaning (Sarda and Desnuelle, 1958). [b] Determined at 40° with monomeric 1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine and with micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine, respectively, and expressed in μequiv min^{-1} mg of protein^{-1} (0.1 M NaCl–0.01 M $CaCl_2$–8 mM acetate buffer (pH 6.0)). [c] AMPA prepared from des[Ala^8]-AMPA or from [Ala^7] AMPA. [d] This value is the maximal, specific activity; a small contamination of L-Ala in the D-Ala, or some racemization during coupling cannot be precluded.

As can be seen from Table I ϵ-amidinated prophospholipase A_2 has about 50% of the enzymatic activity of AMPA using monomeric solutions of L-dihexanoyllecithin. However, when using micellar substrate (L-dioctanoyllecithin) AMPA shows a tremendous increase in enzymatic activity, whereas the ϵ-amidinated zymogen does not. This fundamental difference between ϵ-amidinated prophospholipase A_2 and AMPA, similar to that already described for the native precursor and enzyme, has been attributed to the presence of a so-called interface recognition site (IRS) in AMPA which is not present in the ϵ-amidinated zymogen. It has been shown previously that the presence of the IRS is correlated with the formation of a salt bridge between the N-terminal α-amino group of phospholipase A_2 and probably a buried carboxylate group (Abita et al., 1972; Pieterson et al., 1974; van Dam-Mieras et al., 1975). From the results in Table I it is obvious that AMPA analogues in which the N-terminal α-amino group is blocked, although completely inactive toward micellar substrate just as the zymogen, still have enzymatic activities toward substrate present in the monomeric form comparable to that of AMPA.[8] More remarkable, however, is the fact that [Ala^7]AMPA and des-Ala^8-AMPA, obtained from AMPA by chain elongation and chain shortening, respectively, are also devoid of enzymatic activity toward micellar substrate.[8] Apparently

[5] In addition to the α-amino group the ϵ-amino function is the only reactive group during Edman degradation (Edman, 1950) and chain elongation with activated amino acid esters.

[6] V_{max} values were determined on micellar 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine as described in the legends of Table I. K_{Ca} values were determined as described previously (de Haas et al., 1971).

[7] Although some minor modifications of sensitive amino acid residues like tryptophan, asparagine, and glutamine could have occurred it can be concluded that the procedures do not cause any damage to essential amino acid residues.

[8] It has to be remarked, however, that these N-terminally modified AMPA analogues have considerably higher K_m values for monomeric substrates than AMPA.

these proteins although having free α-amino functions are not able to form the salt bridge. Consequently, these AMPA analogues lacking the IRS are no longer able to interact with lipid–water interfaces as has been demonstrated directly by equilibrium gel filtration using a micellar lipid system (J. C. Vidal, unpublished results).

AMPA analogues in which more than one amino acid residue has been removed like des-(Ala^8,Leu^9)-, des-(Ala^8,Leu^9,Trp^{10})-, and des-(Ala^8–Arg^{13})-AMPA not only are completely devoid of enzymatic activity toward micellar substrate, but have also lost their enzymatic activity toward monomeric substrate. These drastic changes probably have destroyed the active site construction notwithstanding the fact that these proteins still can bind Ca^{2+}.

On the other hand, substitution of the N-terminal L-Ala^8 in AMPA by amino acid residues such as Gly or β-Ala gives rise to the formation of AMPA analogues which not only have rather normal enzymatic activities toward monomeric substrate but also high enzymatic activities toward micellar substrate (Table I). Apparently these AMPA analogues having free N-terminal amino functions allow the formation of the salt bridge and consequently of the IRS.

The sensitivity of the salt bridge formation to minor changes in the N-terminal amino acid residue is demonstrated most clearly by substitution of L-Ala^8 for the stereoisomeric D-Ala residue. Again the active site construction of [D-Ala^8]AMPA turns out to be rather similar to that of AMPA as can be concluded from their similar enzymatic activities toward monomeric substrate. However, in contrast to [Gly^8]- and [β-Ala^8]AMPA the minor change, viz., substitution of L-Ala^8 for D-Ala, impedes the formation of the salt bridge as is evident from the low enzymatic activity of [D-Ala^8]AMPA toward micellar substrate, which is only 2.9% of that of AMPA.

On the other hand, the substitution of Trp^{10} by the hydrophobic Phe residue apparently does not impede formation of the ion pair and [Ala^8,Leu^9,Phe^{10}]AMPA shows considerable interfacial activity (Table I). Also specific chemical modification of Trp^{10} in native phospholipase A_2 by *o*-nitrophenylsulfenyl chloride gives rise to an enzyme with the same interfacial activity as the native enzyme. Apparently the introduction of the bulky NPS group does not perturb the salt bridge formation, suggesting that the Trp residue should possess a rather outwardly exposed position.

It has to be concluded from the above results that the induction of the IRS in phospholipase A_2 requires a very exact juxtaposition of the α-amino group and its negatively charged counterpartner. Work is in progress to further investigate the interaction of the modified enzymes described in this paper with different lipid–water interfaces on a molecular level.

Supplementary Material Available

Amino acid analyses of ϵ-amidinated prophospholipase A_2 (AMPREC), ϵ-amidinated phospholipase A_2 (AMPA) and various N-terminally modified AMPA analogues will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Business Office, Books and Journals Division, American Chemical Society, 1155 16th St., N.W., Washington D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number BIO-75-5394.

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