Asp-49 is not an absolute prerequisite for the enzymic activity of low-M_r phospholipases A_2: purification, characterization and computer modelling of an enzymically active Ser-49 phospholipase A_2, ecarpholin S, from the venom of *Echis carinatus sochureki* (saw-scaled viper)

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Several studies have shown that Asp-49 is the residue that controls calcium binding in, and so plays a critical role in the calcium-mediated activation of, low-M_r group I–III phospholipases A_2 (PLA_2s). The present paper provides experimental evidence that Asp-49 is not an absolute prerequisite for the enzymic activity of PLA_2s, and that proteins with amino acid(s) other than Asp at position 49 can exhibit significant phospholipase activity. The purification, complete amino acid sequence and characterization of ecarpholin S, a PLA_2 from *Echis carinatus sochureki* (saw-scaled viper) venom, is described. This single-chain, 122-amino-acid, basic (pI 7.9) protein is a group II PLA_2. Although Asp-49 is replaced by Ser and Tyr-28 by Phe (both of these positions being involved in the Ca^{2+}-binding site of PLA_2s), the lipolysis of soybean phosphatidylcholine and egg yolk in the presence of 10 mM $CaCl_2$ was 1.5 times and 2.9 times greater respectively with ecarpholin S than with recombinant human group II PLA_2. The Ca^{2+}-dependencies of the enzymic activities of ecarpholin S and $rPLA_2$ were found to be similar. Ecarpholin S added to washed platelets induced aggregation; the presence of Ca^{2+} was a prerequisite for this platelet-aggregating effect. Computer modelling of the Ca^{2+}-binding site of Ser-49 PLA_2 compared with the Asp-49 and Lys-49 forms, for which crystallographic data exist, shows that the Ca^{2+}-binding site is sterically blocked by Lys-49 but not by Ser-49; in the latter, the Ser hydroxy group may replace the Asp carboxylate in stabilization of Ca^{2+} binding. Sequence comparisons of ecarpholin S and other low-M_r PLA_2s predicts the presence of a Ser-49 group in the protein family of low-M_r PLA_2s that is distinct from the Asp-49 and Lys-49 groups.

INTRODUCTION

Phospholipase A_2 (PLA_2; EC 3.1.1.4) hydrolyses the *sn*-2 fatty acyl ester bond of glycerophospholipids, producing non-esterified fatty acids and lysophospholipids (reviewed in [1]). Based on their amino acid sequences, PLA_2s have been divided into main groups and subgroups (reviewed in [2]). The enzymic activity of low-M_r PLA_2s is absolutely dependent on the presence of calcium ions. Residues 26–34 and 49, comprising the Ca^{2+}-binding domain, and especially amino acids Tyr-28, Gly-30, Gly-32 and Asp-49, which are directly involved in calcium binding by providing oxygens, are extremely well conserved in PLA_2s [3–6] (numbering corresponds to that proposed by Renetseder et al. [7]).

Since 1984 [8], PLA_2-like proteins containing Lys-49 instead of Asp-49 have been isolated and characterized from various snake venoms [9–12]. At first, there was disagreement in the literature as to whether Lys-49 PLA_2s are enzymically inactive or have little phospholipase activity. Later, studies with high-purity Lys-49 PLA_2s suggested that the low enzymic activity measured in earlier studies was the result of contamination with Asp-49 PLA_2s, and site-specific mutagenesis [13,14] and crystallographic studies [15,16] provided a molecular basis for the lack of enzymic activity of Lys-49 molecules. It is now generally accepted that Asp-49 is an absolute prerequisite for Ca^{2+} binding by, and hence enzymic activity of, low-M_r PLA_2s. Ammodytin L, a myotoxic PLA_2 homologue from *Vipera ammodytes* venom, has been found to contain a Ser residue at position 49 replacing the highly conserved Asp [17]. Because of this and two other mutations in the calcium-binding site (Tyr-28 to His and Gly-33 to Asp), the low PLA_2 activity of ammodytin L was considered in a previous report to be due to contamination with ammodytoxins [18], without examination of the enzymic properties of a high-purity sample [17]. All of the Lys-49 PLA_2s and the Ser-49 protein, ammodityn L, are group II PLA_2s. The only group I PLA_2 that has an amino acid other then Asp at position 49, Ala-49 PLA_2 from *Bungarus fasciatus* (Bfas-A49) [19], is reported to be enzymically inactive. Thus all of the naturally occuring PLA_2 homologues with a mutation at position 49 that have been previously reported are considered to be enzymically inactive.

Here we describe the purification, characterization (including the complete amino acid sequence), computer modelling of the Ca^{2+}-binding loop and sequence comparison with other PLA_2s of ecarpholin S, an enzymically active Ser-49 PLA_2 from *Echis carinatus sochureki* (saw-scaled viper) venom.

MATERIALS AND METHODS

Purification and *S*-pyridylethylation of ecarpholin S

Lyophilized *Echis carinatus sochureki* venom (Latoxan, Rosans, France) was dissolved at 10 mg/ml in 50 mM sodium acetate, pH 5.0. Insoluble components were removed by centrifugation and the supernatant was loaded on to a Fractogel EMD SO^{3-} cation-exchange column (10 mm × 150 mm; Merck, Darmstadt,

Abbreviations used: PLA_2, phospholipase A_2; $rPLA_2$, recombinant human group II PLA_2; non-Asp-49 PLA_2s, PLA_2s having an amino acid other than Asp at position 49; *S*-PE-ecarpholin S, *S*-pyridylethylated ecarpholin S; TFA, trifluoroacetic acid; Bfas-A49, Ala-49 PLA_2 from *Bungarus fasciatus* (inactive); App-K49, *Agkistrodon piscivorus piscivorus* Lys-49 PLA_2; Myo-II, *Bothrops asper* myotoxin II; Nna-PLA_2, *Naja naja atra* PLA_2.

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The protein sequence reported in this paper has been deposited in the PIR database under accession no. P48650.

Germany). The column was extensively washed with starting buffer before applying a linear gradient of 0–1 M NaCl in 50 mM sodium acetate, pH 5.0, at a flow rate of 2 ml/min. Fractions were assayed for their ability to induce platelet aggregation. The pH of 100 μl probes was corrected to 7.4 by adding 5 μl of 1 M Tris, pH 8.0, before the aggregation studies. Fractions (2 ml) which induced platelet aggregation from several preparations were eluted with 0.35–0.4 M NaCl gradients, pooled, dialysed, dissolved in 0.1 % trifluoroacetic acid (TFA) and further purified by reverse-phase HPLC on a wide-pore C4 column (4.6 mm $\times$ 250 mm; J. T. Baker, Phillipsburg, NJ, U.S.A.). Aliquots of 1/20 vol. of the fractions eluted with an acetonitrile gradient (0.1 % TFA) were lyophilized, dissolved in 145 mM NaCl/50 mM Tris, pH 7.4, and assayed for platelet aggregation-inducing activity. Active fractions, eluting at approx. 35 % acetonitrile, were pooled from several runs, lyophilized, dissolved in 0.1 % TFA and rechromatographed on a C4 column. The main peak fractions from several runs, representing purified ecarpholin S, were pooled, lyophilized and stored at 4 °C.

To 1 vol. of 300 μg/ml ecarpholin S in 6 M guanidine hydrochloride/0.1 M Tris, pH 8.0, was added 1/20 vol. of 440 mM dithiothreitol, followed by incubation at 45 °C for 30 min. Then 1/20 vol. of 1 M 4-vinylpyridine was added, and after a 1 h incubation at room temperature the sample was diluted with 2 vol. of 0.2 % TFA. Modified ecarpholin S was isolated free of reagents by reverse-phase HPLC as described above.

Sequencing strategy

Sequence analysis was performed on an Applied Biosystem model 477A pulsed-liquid-phase protein sequencer with a model 120A on-line phenylthiohydantoin amino acid analyser. N-terminal sequencing of *S*-pyridylethylated ecarpholin S (S-PE-ecarpholin S) yielded 30 residues from the N-terminus. Aliquots of 20 μg of S-PE-ecarpholin S were used for enzymic and CNBr digestions. Endoproteinase Asp-N (Boehringer Mannheim; 2 % by mass) was added to the protein dissolved in 100 μl of 100 mM Tris/HCl, pH 8.0, and incubated at 37 °C for 18 h. Endoproteinase Glu-C (*Staphylococcus aureus* V8; Boehringer Mannheim; 3.5 % by mass) was added to the protein dissolved in 100 μl of 25 mM NH_4HCO_3, pH 7.8, and incubated at 37 °C for 18 h. For pepsin digestion, the protein was dissolved in 100 μl of 3.5 % formic acid and 2 μg of pepsin (Sigma; 10 % by mass) was added; the solution was incubated for 18 h at room temperature. For CNBr cleavage, the protein was dissolved in 100 μl of 70 % formic acid containing 10 mg/ml CNBr and incubated for 18 h in the dark at room temperature. The peptides generated by proteolytic and CNBr digestions were separated by reverse-phase HPLC (Hewlett Packard HP1090), using a Brownlee C18 column (220 mm $\times$ 2.1 mm). The peptides were eluted with an acetonitrile gradient (0.1 % TFA) from 0 to 50 % over 60 min, followed by 50 to 65 % over 5 min. The elution was monitored using a diode-array detector at 214 nm and 280 nm, and all of the peaks, collected manually, were sequenced.

Amino acid analysis

A 1 nmol sample of protein was gas-phase-hydrolysed under vacuum in a nitrogen atmosphere for 24 h in 6 M HCl containing 1 mg/ml phenol at 112 °C. The resultant amino acids were quantified using Waters AccQ.Tag chemistry and a Hewlett Packard HP1090 liquid chromatograph.

Electrospray MS

Electrospray mass spectra were obtained using a Trio 2/3000 ESI instrument (VG Biotech). Samples from reverse-phase HPLC (TFA/acetonitrile system) were directly injected into the solvent stream of methanol/water/acetic acid (49.5:49.5:1, by vol.), or were dried and then dissolved in this solvent system, and were infused at a rate of 2 μl/min. Spectra were co-added by repetitive scanning until an acceptable signal-to-noise ratio was obtained.

Computer analysis of sequence data

Computer analysis of the sequence data was performed on a VAX/VMS system using the suite of programs from the University of Wisconsin Genetics Computer Group. Alignments were carried out using the program PILEUP, with a gap-creating penalty of 2.0 and a gap extension penalty of 0.1.

Protein determination

Protein determination was performed using the BCA protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.) with BSA as a standard.

SDS/PAGE and silver staining

SDS/PAGE was performed according to Laemmli [20], and the gels were silver-stained by the method of Morrissey [21].

Preparation of washed platelets and platelet aggregation

Human platelets were isolated from buffy coats less than 20 h after blood collection; buffy coats were obtained from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service. To one buffy coat was added 30 ml of 100 mM citrate, pH 6.5. Platelet-rich plasma and the platelet pellet were isolated by successive centrifugation steps. Platelets were resuspended in a small volume of 113 mM NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.4 mM NaH_2PO_4 and 5.5 mM glucose, pH 6.5 (buffer A). Iloprost (a gift from Schering A. G., Zürich, Switzerland) (10 μl; 0.5 μg/ml) was added, and the suspension was diluted to 40 ml with buffer A and centrifuged at 250 ***g*** for 5 min. The platelet-rich supernatant was centrifuged at 1000 ***g*** for 10 min, and platelets were washed twice with buffer A. Washed platelets were resuspended in 20 mM Hepes, 140 mM NaCl and 5.5 mM glucose, pH 7.4 (buffer B), and the platelet count was adjusted to 10^9 platelets/ml by dilution with buffer B. Samples were kept at room temperature until used for aggregation studies. Platelet aggregation was monitored by light transmission [22] using a Lumitec aggregometer with continuous stirring at 1300 rev./min at 37 °C. Platelets were preincubated in buffer containing 2 mM $CaCl_2$ at 37 °C for 2 min before starting the measurement by adding the samples for analysis.

Recombinant human group II PLA_2 ($rPLA_2$)

$rPLA_2$ [23] was kindly provided by Dr. Ruth M. Kramer (Cardiovascular Research, Lilly Research Laboratories, Indianapolis, IN, U.S.A.).

Measurement of phospholipase enzymic activity

Phospholipase enzymic activity was measured by the titration method of Nieuwenhuizen et al. [24], using egg yolk as a substrate, and by the colorimetric assay of de Araujo and Radvanyi [25], with L-phosphatidylcholine (Type IV-S; Sigma Chemical Co.) as substrate, at room temperature.

Computer modelling

The three-dimensional structure of ecarpholin S was modelled using comparative protein modelling methods as implemented in the automated protein modelling server Swiss-Model [26,27]. Beforehand, a database of sequences derived from known protein structures was searched with the ecarpholin S sequence using BLASTP [28]. The two most similar PLA_2s with crystallographic data available, *Agkistrodon piscivorus piscivorus* Lys-49 PLA_2 (App-K49) [15] and *Bothrops asper* myotoxin II (Myo-II) [29] [Brookhaven Protein Data Bank (PDB) entry codes 1PPA and 1CLP], were selected as modelling templates. The sequence of ecarpholin S was submitted to Swiss-Model along with the PDB entry codes of the two modelling templates. The ecarpholin S model and the Myo-II structure were superimposed on to the co-ordinates of *Naja naja atra* PLA_2 (Nna-PLA_2) [5] (PDB entry 1POB), which was co-crystallized with a substrate transition state analogue and calcium. This operation allowed the identification of both substrate- and calcium-binding sites in ecarpholin S and Myo-II. Further optimization of the ecarpholin S model, especially to reflect the conformation of the Tyr-28 to Gly-32 loop of the substrate/calcium-bound state of Nna-PLA_2, was carried using the Optimize mode of the Swiss-Model server. A multiple sequence alignment, which included Nna-PLA_2 as a template, was submitted to the server. The resulting final model structure was inspected for sequence-to-structure fitness using both 3D/1D profiles [30] and ProsaII [31].

RESULTS

Purification and sequence analysis of ecarpholin S

Ecarpholin S was purified from *Echis carinatus sochureki* venom by a three-step procedure on cation-exchange and reverse-phase chromatography columns (Figure 1). From 1 g of lyophilized venom, which contained 100 mg of protein soluble in 50 mM sodium acetate, pH 5.0, 3 mg of ecarpholin S could be purified by this method. The isolated protein has an apparent M_r of 15000 (non-reduced)/15500 (reduced), as analysed by SDS/PAGE and Coomassie Blue or silver staining (results not shown). No additional protein bands could be detected in the gel by either of these staining methods.

The amino acid sequence of ecarpholin S (Figure 2) was obtained from intact S-PE-ecarpholin S, as well as from peptide fragments after proteolytic cleavage of S-PE-ecarpholin S with CNBr and endoproteinases Asp-N, Glu-C and pepsin. All peptide sequences were consistent with the structure presented. All residues were unambiguously determined by direct sequencing of intact S-PE-ecarpholin S and peptides derived from several digests of S-PE-ecarpholin S. Ecarpholin S consists of 122 amino acids. The isoelectric point and the molar absorption coefficient of ecarpholin S, calculated from its amino acid sequence, are 7.88 and 18050 $M^{-1} \cdot cm^{-1}$ respectively. A good correlation was found between the amino acid composition calculated from the sequence and that measured (Table 1). Electrospray MS analysis of ecarpholin S showed that the protein that represents more than 99.5% of the sample has an M_r of 13805 ± 2.7 (mean $\pm$ S.D., $n = 3$). This is in good agreement with the M_r of ecarpholin S of 13804 calculated from the amino acid sequence.

Sequence comparison of ecarpholin S and other low-M_r PLA_2s

Ecarpholin S belongs to the PLA_2 protein family (group II; subgroup IIA) and shows strong sequence similarity to group I–II PLA_2s. The best similarity, 66% identity in a 122-amino-acid overlap, was found with *Vipera ammodytes* ammodytin L

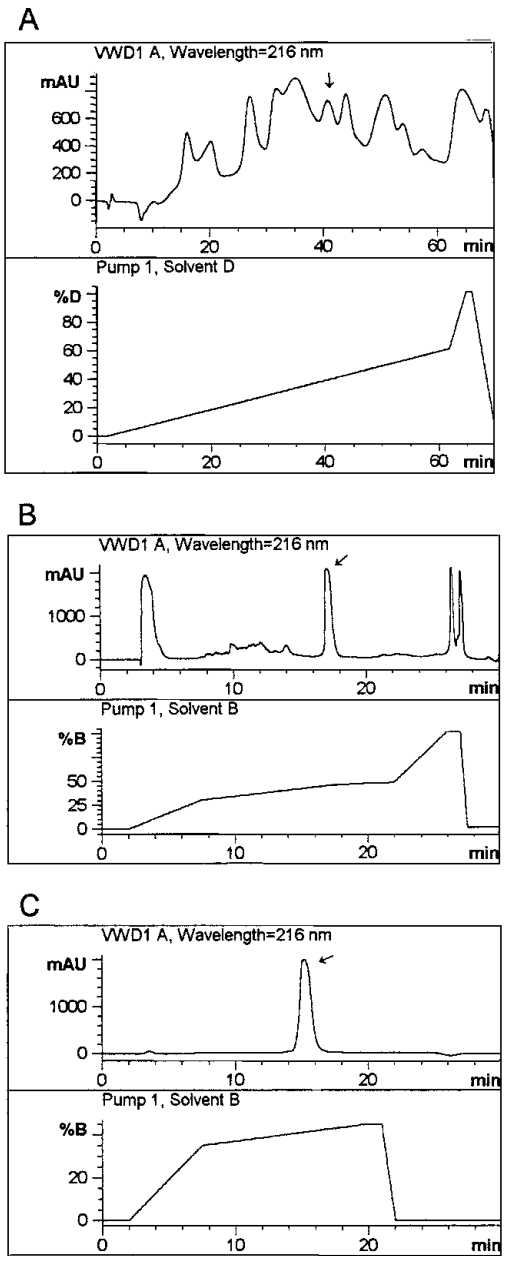

Figure 1 Purification of ecarpholin S from *Echis carinatus sochureki* venom

(**A**) Typical chromatogram of cation-exchange chromatography on a Fractogel EMD SO^{3-} column. Also shown are typical chromatograms of the first (**B**) and the second (**C**) reverse-phase HPLC steps on a C4 column. mAU, milli-absorbance units at 216 nm; %D, percentage of 1 M NaCl; %B, percentage of 80% acetonitrile. Arrows indicate protein peaks containing ecarpholin S (for details, see the Materials and methods section).

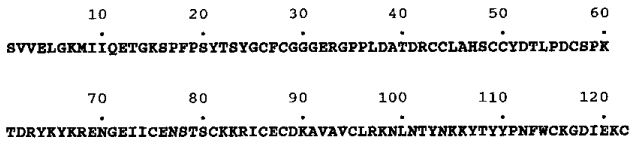

Figure 2 Amino acid sequence of ecarpholin S obtained from *Echis carinatus sochureki* venom

Table 1 Amino acid composition of ecarpholin S

The calculated values were obtained from the primary sequence. Measured values are means ± S.D.; $n = 3$.

Amino acid	No. of residues	
	Calculated	Measured
Asx	13	12.70 ± 0.18
Ser	8	7.88 ± 0.04
Glx	9	8.91 ± 0.07
Gly	9	8.84 ± 0.04
His	1	1.14 ± 0.05
Arg	6	6.08 ± 0.04
Thr	8	7.82 ± 0.16
Ala	4	3.90 ± 0.04
Pro	7	6.68 ± 0.06
Cys	14	12.75 ± 0.03
Tyr	9	9.09 ± 0.11
Val	4	3.60 ± 0.02
Met	1	0.89 ± 0.09
Lys	13	13.31 ± 0.13
Ile	6	4.77 ± 0.01
Leu	6	5.99 ± 0.04
Phe	3	2.98 ± 0.02
Trp	1	0.71 ± 0.11

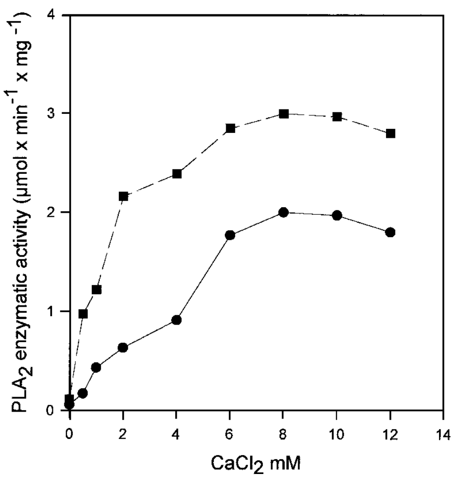

Figure 4 Ca^{2+}-dependency of the enzymic activities of ecarpholin S and rPLA$_2$

Enzymic activity (μmol/min per mg of protein) in the presence of various concentrations of $CaCl_2$ was measured by the colorimetric method of de Araujo and Radvanyi [25] at 25 °C. ■, Ecarpholin S; ●, rPLA$_2$.

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                   +     ++  +                                   O          O
             1   * *    **** ** 20   *    *          *40         *       *  *          70
Ecs-S49      SVVELGKMII QETG.KSPFP SYTSYGCFCG GGERGPPLDA TDRCCLAHSC CY...DTLPD C.....SPKT
Amm.L        SVIEFGKMIQ EETD.KNPLT SYSFYGCHCG LGNKGKPKDA TDRCCFVHSC CY...AKLPD C.....SPKT
App-K49      SVLELGKMIL QETG.KNAIT SYGSYGCNCG WGHRGQPKDA TDRCCFVHKC CY...KKLTD C.....NHKT
ACLMT        SLLELGKMIL QETG.KNAIT SYGSYGCNCG WGHRGQPKDA TDRCCFVHKC CY...KKLTD C.....NHKT
Myo-II       SLFELGKMIL QETG.KNPAK SYGAYGCNCG VLGRGKPKDA TDRCCYVHKC CY...KKLTG C.....NPKK
Bthtx-I      SLFELGKMIL QETG.KNPAK SYGAYGCNCG VLGRGKPKDA TDRCCYVHKC CY...KKLTG C.....NPKK
BP-I         SLVQLWKMIF QETG.KEAAK NYGLYGCNCG VGRRGKPKDA TDSCCYVHKC CY...KKVTG C.....DPKM
BP-II        SLVQLWKMIF QETG.KEAAK NYGLYGCNCG VGRRGKPKDA TDSCCYVHKC CY...KKVTG C.....NPKM
TMV-K49      SLIELGKMIF QETG.KNPVK NYGLYLCNCG VGNRGKPVDA TDRCCFVHKC CY...KKVTG C.....DPKM
Tgr-K49      SVIELGKMIF QETG.KNPAT SYGLYGCNCG PGGRRKPKDA TDRCCYVHKC CY...KKLTD C.....DPIK
Ba-K-49      SLVELGKMIL QETG.KNPLT SYGVYGCNCG VGSRHKPKDD TDRCCYVHKC CY

Bfas-A49     NMVQFKSMV. QCTS.TRPWL DYVDYGCNCD IGGTGTPLDE LDRCCQTHAN CYTEARKFPE C.....APYY


                   OO O        O    O+                 +  + +++           +O
             *  *  ** *      * *    **    100  *   * * *  * *** 120       **
Ecs-S49      DRYKYKRENG EIIC.ENSTS CKKRICECDK AVAVCLRKNL NTYNKKYTYY PN.FWCKGDI EKC
Amm.L        NRYEYHRENG AIVC.GSSTP CKKQICECDR AAAICFRENL KTYNKKYKVY LR.FKCKGVS EKC
App-K49      DRYSYSWKNK AIICEEKN.P CLKEMCECDK AVAICLRENL DTYNKKYKAY FK.LKCKK.P DTC
ACLMT        DRYSYSWKNK AIICEEKN.P CLKEMCECDK AVAICLRENL DTYNKKYKAY FK.FKCKK.P ETC
Myo-II       DRYSYSWKDK TIVCGENN.S CLKELCECDK AVAICLRENL NTYNKKYRYY LKPLFCKK.A DAC
Bthtx-I      DRYSYSWKDK TIVCGENN.P CLKELCECDK AVAICLRENL GTYNKKYRYH LKP.FCKK.A DPC
BP-I         DSYSYSWKNK AIVCGEKNPP CLKQVCECDK AVAICLRENL GTYNKKYTIY PKPF.CKK.A DTC
BP-II        DSYSYSWKNK AIVCGEKNPP CLKQVCECDK AVAICLRENL GTYNKKYTIY PKPF.CKK.A DTC
TMV-K49      DRYSYSWENK AIVCGEKNPP CLKQVCECDK AVAICLRENL QTYDKKHRVT VKFL.CKA.P ESC
Tgr-K49      DRYSYSWVNK AIVCGEDN.P CLKEMCECDK AVAICFRENL DTYDKKKKIN LK.LFCKKTS EQC

Bfas-A49     KTYSYTCSGG TITCNADNDE CAASVCNCDR TAALCFAG.. APYNQNNFDV DLETRCQ
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Figure 3 Sequence comparison of ecarpholin S and other non-Asp-49 PLA$_2$s

Residues conserved in Lys-49 PLA$_2$s, but different from those in Asp-49 PLA$_2$s, according to de Araujo et al. [32], are indicated by asterisks. Residues conserved in both Lys-49 and Ser-49 PLA$_2$s, but different from those in Asp-49 PLA$_2$s, are indicated by +. Residues where amino acids conserved in Lys-49 PLA$_2$s are replaced by the same amino acid in both of the Ser-49 PLA$_2$s are indicated by circles. Ser residues proposed to be uniquely characteristic of Ser-49 PLA$_2$s are underlined. Amino acid sequences are: *Echis carinatus sochureki* ecarpholin S (Ecs-S49), *Vipera ammodytes ammodytes* ammodytin L (Amm.L) [17], App-K49 [9], *Agkistrodon contortrix laticinctus* myotoxic Lys-49 PLA$_2$ (ACLMT) [32], Myo-II [12], *Bothrops jararacussu* bothropstoxin I (Bthtx-I) [33], *Trimeresurus flavoviridis* basic protein I (BP-I) [10], *T. flavoviridis* basic protein II (BP-II) [11], *Trimeresurus mucrosquamatus* Lys-49 PLA$_2$ (TMV-K49) [34], *Trimeresurus gramineus* myotoxic Lys-49 PLA$_2$ (Tgr-K49) [35]; *Bothrops atrox* Lys-49 PLA$_2$, partial sequence (Ba-K49) [8], and Bfas-A49 [19]. Alignments were carried out using the program PILEUP, with a gap-creating penalty (gcp) of 1.2 and a gap extension penalty of 0.1. Note that a gcp larger than 1.2 resulted in different alignment in the C-terminal region, starting from position 122, of the proteins. Using a gcp larger than 1.2 or 1.8, the conserved Cys-126 of Tgr-K49, and, in addition, Ecs-49 and Amm. L, did not overlap with the corresponding Cys residues of the other proteins. The positions are numbered as proposed by Renetseder et al. [7].

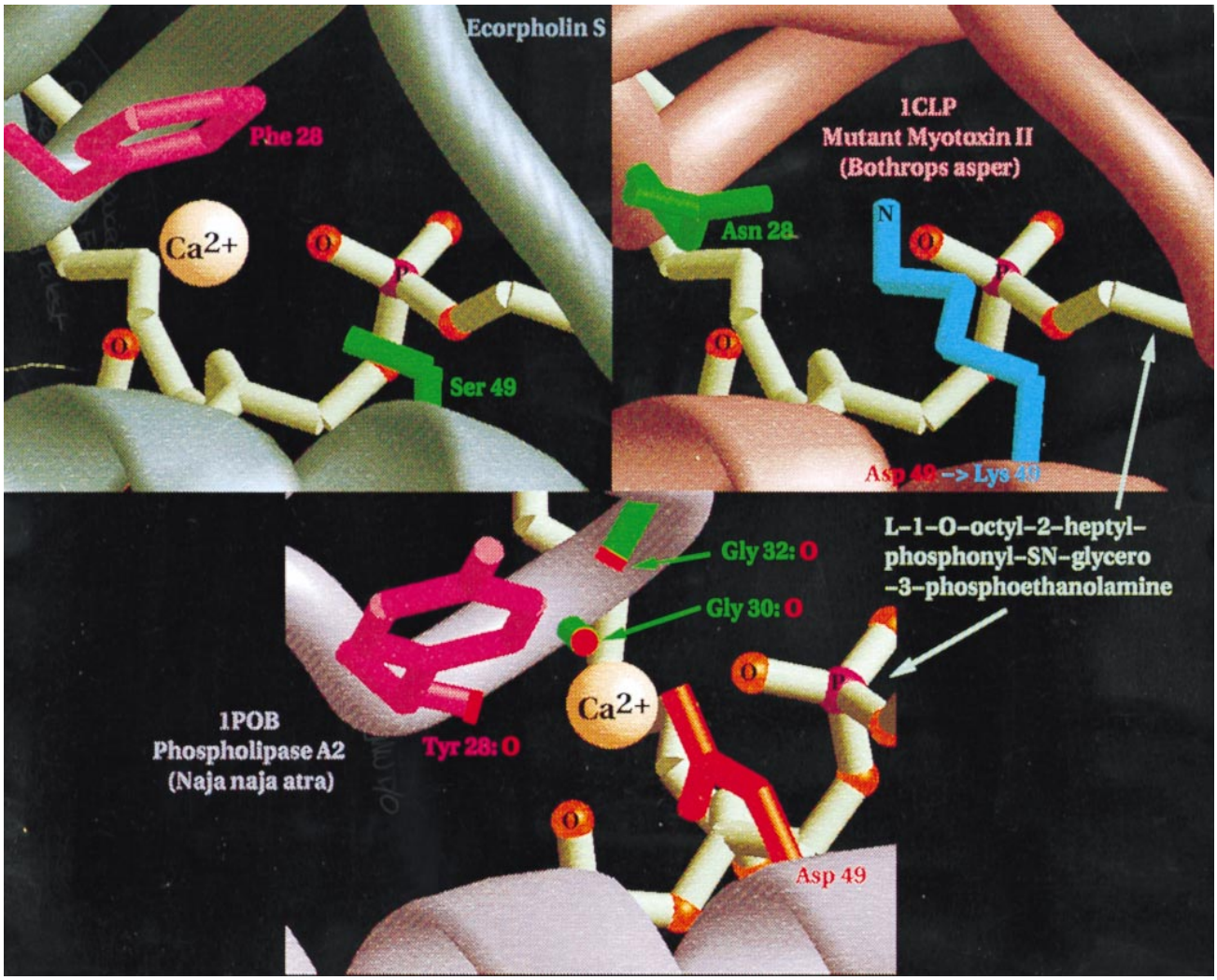

Figure 5 Three-dimensional structures of PLA_2s

The model of ecarpholin S (upper left) and the structure of Myo-II (one of the modelling templates; Brookhaven Protein Data Bank entry code 1CLP; upper right) were superimposed on the structure of Nna-PLA_2 (PDB entry code 1POB; lower panel). The spatial location of the transition-state analogue (L-1-*O*-octyl-2-heptylphosphonyl-*SN*-glycero-3-phosphoethanolamine) and the calcium ion could thereby help to identify the substrate- and calcium-binding locations for both ecarpholin S and Myo-II. In ecarpholin S, the calcium-binding site may be formed by backbone carbonyl oxygens and the side chain of Ser-49, in addition to the substrate's phosphate group, much like the situation in Nna-PLA_2 (lower panel). In contrast, Myo-II does not bind calcium, since the ϵ-amino group of Lys-49 (noted as N) occupies the portion of space normally taken by the calcium ion.

[17], the only Ser-49 PLA_2 described previously. Sequence comparisons of ecarpholin S with other low-M_r PLA_2s having an amino acid other than Asp at position 49 (non-Asp-49 PLA_2s) described so far [8–12,17,19,32–35] are shown in Figure 3. Residues that have been shown to be conserved in Lys-49 PLA_2s and to distinguish Lys-49 PLA_2s from Asp-49 PLA_2s [32] are indicated (asterisks). There are 33 such residues. In 11 of these 33 positions, both ecarpholin S and the other Ser-49 PLA_2, ammodytin L, have amino acids characteristic of Lys-49 PLA_2s (Figure 3, +). These residues represent similarities between Lys-49 PLA_2s and Ser-49 PLA_2s, and distinguish Ser-49 PLA_2s from Asp-49 PLA_2s. The fact that there are only 11 such residues out of 33 shows that the two Ser-49 PLA_2s do not fit properly into the group of Lys-49 PLA_2s. In addition, there are seven positions out of 33 where amino acids conserved in Lys-49 PLA_2s are replaced by the same amino acid in both of the Ser-49 proteins (Figure 3, circles). Furthermore, three Ser residues present in ecarpholin S and ammodytin L (Figure 3, underlined) are unique to low-M_r PLA_2s. Neither Lys-49 proteins nor the 40 PLA_2s aligned in the paper of Davidson and Dennis [6] have Ser at positions 49 and 88, and only two of them have Ser-67. The only known Ala-49 PLA_2, inactive Bfas-A49 [19], lacks those conserved residues that are characteristic of Lys-49 and/or Ser-49 PLA_2s.

Phospholipase enzymic activity of ecarpholin S

Because of the Asp-49 → Ser and Tyr-28 → Phe substitutions, both at positions that are involved in the calcium-binding site of PLA_2s, ecarpholin S was expected to be enzymically inactive. However, surprisingly, ecarpholin S was found to be active. Its phospholipase enzymic activity, compared with the activity of rPLA_2, was $149 \pm 12\%$ ($n = 5$) measured with soybean phosphatidylcholine/deoxycholate at room temperature by the

colorimetric assay of de Araujo and Radvanyi [25], and $293 \pm 27\%$ ($n = 5$) with egg yolk substrate at room temperature analysed by the titration method of Nieuwenhuizen et al. [24].

A comparison of the Ca^{2+}-dependency of the enzymic activities of ecarpholin S and $rPLA_2$ is shown in Figure 4. The enzymic activities of the Ser-49 ecarpholin S and the Asp-49 $rPLA_2$ are dependent on Ca^{2+} to the same extent, reaching a maximum activity at about 6–10 mM added $CaCl_2$ in both cases.

Purified ecarpholin S at low (μg/ml) concentrations added to washed platelets induced aggregation (results not shown). The presence of Ca^{2+} (optimal concentration 2–4 mM) is a prerequisite for the platelet-aggregating effect of ecarpholin S.

Molecular model of ecarpholin S

A molecular model of ecarpholin S (Figure 5) was derived from three known PLA_2 structures using comparative modelling methods as implemented in the automated protein modelling server Swiss-Model [26,27]. The sequence-to-structure fitness of the model was assessed by a 3D/1D profile [30] (average score per residue was 0.46) and ProsaII [31]. No region of the model showed structural inconsistencies with either method, indicating the global soundness of the model.

The calcium-binding site of PLA_2s, as seen in the crystal structure of Nna-PLA_2 is formed by the side chain of Asp-49, the backbone carbonyl oxygens of Tyr-28, Gly-30 and Gly-32, and the phosphate group of the phospholipid substrate (all distances less than 2.5 Å). As no sequence insertions or deletions occur in this region, it is reasonable to assume that the structure of ecarpholin S will be closely similar to that of Nna-PLA_2. The superposition of the structures of several PLA_2s on to the structure of Nna-PLA_2 reveals that this region changes its conformation upon calcium binding, apparently to optimize the polar interactions between the calcium ion and the protein side chain and backbone groups described above. Our comparative model of ecarpholin S was thus corrected in this region, to reflect the bound state.

The structural basis for the loss of enzymic activity of Lys-49 PLA_2s can be easily explained by the spatial location of the ϵ-amino group of Lys-49 (Figure 5). Indeed, its extended side chain reaches into the portion of space normally occupied by the calcium ion. In addition to a charge incompatibility, steric hindrance is therefore responsible for the lack of calcium binding by this class of PLA_2s. Ecarpholin S, in contrast, can accommodate a calcium ion at this site, not only because of the short side chain of Ser-49 but also due to the polar character of its side-chain hydroxy group. All of the backbone carbonyl oxygens present in Nna-PLA_2, as well as the substrate's phosphate group, also participate in calcium co-ordination by ecarpholin S.

DISCUSSION

The purification, complete amino acid sequence and characterization of ecarpholin S, a 122-amino-acid group II PLA_2 from *Echis carinatus sochureki* venom, is described. In spite of two substitutions, Asp-49 to Ser and Tyr-28 to Phe, in the extremely well conserved Ca^{2+}-binding site of PLA_2s, ecarpholin S showed significant phospholipase enzymic activity and Ca^{2+}-dependent platelet-aggregating activity. Because an Asp-49 isoenzyme of ecarpholin S is not yet known, the enzymic activity of ecarpholin S was compared with that of another well characterized group II PLA_2, $rPLA_2$. The lipolysis of soybean phosphatidylcholine and egg yolk in the presence of 10 mM $CaCl_2$ was 1.5 times and 2.9 times greater respectively with ecarpholin S than with $rPLA_2$. The Ca^{2+}-dependencies of the enzymic activities of ecarpholin S and $rPLA_2$ were found to be similar. Early papers reported low enzymic activity of Lys-49 and Ser-49 PLA_2s. This low enzymic activity, representing ~ 1–5% of the activity of the Asp-49 homologues, was considered to be due to contamination with Asp-49 PLA_2s, and the contaminating species could be resolved by additional chromatographic steps [16,17]. It should be emphasized that the PLA_2 enzymic activity of the ecarpholin S sample used in the present study cannot originate from contaminating Asp-49 PLA_2s. First, the results of SDS/PAGE with Coomassie Blue/silver staining, sequence analysis, amino acid composition and especially electrospray MS clearly show that a high-purity ecarpholin S sample was used. Secondly, traces of Asp-49 PLA_2(s) cannot be responsible for the significant PLA_2 enzymic activity of ecarpholin S, which was found to be higher than the activity of $rPLA_2$ in both assay systems used for activity measurements. These results leave no doubt that ecarpholin S is an enzymically active PLA_2.

Computer modelling of the Ca^{2+}-binding site of ecarpholin S and of Asp-49 and Lys-49 PLA_2s demonstrates that the three-dimensional structures of the binding sites of Asp-49 PLA_2s and ecarpholin S are similar. Whereas in Lys-49 proteins the long ϵ-amino side chain of the Lys residue, which extends into the Ca^{2+}-binding loop and has a positive charge, interferes with correct Ca^{2+} binding, there is no steric inhibition in the case of ecarpholin S, and the Ser-49 hydroxy group provides a partial negative charge, similar to the Asp-49 carboxylate, for stabilizing Ca^{2+} binding. The significant difference between the Ca^{2+} binding of the native (Asp-49) and Glu-, Gln-, Ala- and Lys-49 mutant PLA_2s [13,14] shows that the length and the charge of the side chain of residue 49 are very important parameters. In this respect Ser-49, which has not been examined in site-directed mutagenesis studies, is a potential candidate for replacing Asp-49, because of its hydroxy group and 'not too long' side chain. The results presented here suggest that correct positioning of Ca^{2+} in the Ca^{2+}-binding loop in ecarpholin S is possible. A structural change in the Ca^{2+}-binding loop has a direct effect on the Ca^{2+} dissociation constant (K_d) and the kinetic parameters of the enzymic reaction (k_{cat}, K_m) catalysed by PLA_2s [36]. The question as to whether ecarpholin S has the same K_d, k_{cat} and K_m parameters as its Asp-49/Tyr-28 isoenzyme remains open.

Ecarpholin S is the eleventh non-Asp-49 group II PLA_2 sequenced so far (Figure 3). Asp-49 is replaced by Lys in eight Lys-49 proteins, by Ser in ecarpholin S and ammodytin L, and by Ala in Bfas-A49 (Figure 3). Interestingly, in all non-Asp-49 PLA_2s at least one other amino acid in addition to Asp-49 is changed out of the four that are directly involved in calcium ion binding and are extremely well conserved in Asp-49 PLA_2s (Tyr-28, Gly-30, Gly-32 and Asp-49). Tyr-28 is replaced by Asn in the Lys-49 proteins and Bfas-A49, and by His and Phe in ammodytin L and ecarpholin S respectively. In Myo-II (Leu-32), *Bothrops jararacussu* bothropstoxin-I (Leu-32) and Bfas-A49 (Asp-30), a third amino acid of the four is changed. The recent paper of de Araujo et al. [32] put this unusually high frequency of mutations in the conserved Ca^{2+}-binding loop in the case of Lys-49 PLA_2s in a new light. Based on sequence comparisons of seven Lys-49 PLA_2s with Asp-49 PLA_2s, it has been pointed out that Lys-49 PLA_2s are distinct from the Asp-49 PLA_2s. Lys-49 PLA_2s have several invariant residues not found in Asp-49 PLA_2s and show significantly greater identity to each other than to Asp-49 PLA_2s. Ammodytin L, the only Ser-49 PLA_2 known previously, was found not to have some of the conserved residues of Lys-49 proteins.

Sequence comparisons of all of the non-Asp-49 PLA_2s described so far and ecarpholin S allow some observations. First,

both *Trimeresurus gramineus* myotoxic Lys-49 PLA_2 [35] and *Bothrops atrox* Lys-49 PLA_2 [8], Lys-49 proteins additional to those examined by de Araujo et al. [32], fit into the group of Lys-49 PLA_2s, supporting the proposal that these proteins are members of a distinct protein family. Secondly, variation at the highly conserved positions 28, 30, 32 and 49 is not a unique characteristic of group II PLA_2s. Bfas-A49, an Ala-49 protein lacking enzymic activity [19], is a group I PLA_2. Its sequence differs significantly from those of other non-Asp-49 and Asp-49 proteins. Whether Bfas-49 is the only non-Asp-49 protein in group I is not yet known. Thirdly, the similarities and differences between the amino acid sequences of various PLA_2s described in the Results section, and especially in the case of the two Ser-49 proteins ecarpholin S and ammodytin L (for which the best sequence similarity was found with each other, and which contain three Ser residues, Ser-49, -67 and -88, that are unique among PLA_2s), may predict the existence of a Ser-49 group of proteins distinct from the Lys-49 and Asp-49 groups.

This study shows that ecarpholin S has Ca^{2+}-dependent phospholipase enzymic activity. The other Ser-49 PLA_2, ammodytin L, has been reported previously to have enzymic activity [18], which was later considered to be due to contamination with ammodytoxins [17]. However, this consideration was based on the fact that Asp-49 and two other amino acids in the calcium-binding site are mutated and that site-specific mutagenesis [13] and crystallographic studies [15] provided a molecular basis for the lack of enzymic activity of Lys-49 molecules, but not on activity measurements with high-purity ammodytin L [17]. The enzymic activity of the Ser-49 ecarpholin S suggests that Ca^{2+}-dependent phospholipase enzymic activity of ammodytin L cannot be ruled out.

In a recent paper [37], *Trimeresurus flavoviridis* basic proteins I and II, both Lys-49 PLA_2s, were found to be inactive with monomers and micelles of synthetic phospholipids, as expected, but showed significant enzymic activity with membrane phospholipids. These proteins differ only in amino acid 67, which is Asp and Asn in basic proteins I and II respectively. The significant difference in their enzymic activity towards membrane phospholipids [37] emphasizes the importance of this residue, residue 67, in the PLA_2–membrane interactions. Additional structural data, including the membrane-bound conformation of PLA_2s, are needed to clarify this. It is interesting that all Lys-49 and almost all Asp-49 PLA_2s have Asp or Asn in this position. This also suggests that ecarpholin S and ammodytin L, both with Ser-67, are the first two members of a distinct class of group II PLA_2s.

The quite large (and still increasing) number of low-M_r PLA_2s with known amino acid sequences, and in several cases with known crystallographic data as well, together with the fact that different low-M_r PLA_2s show a wide spectrum of different pharmacological/pathophysiological effects such as myotoxicity, cardiotoxicity, neurotoxicity, and oedema-inducing, convulsive, anticoagulant and platelet effects (reviewed in [38,39]), make this protein family of low-M_r PLA_2s an excellent model system for studying structure–function relationships. There is no direct relationship between the phospholipase enzymic activity and the toxicity of PLA_2s (reviewed in [38]), and specific receptors for PLA_2s have been found on a number of different cells [40–45]. The so-called M-type receptor for PLA_2s [46] has recently been cloned [42,47] and found to be related to the macrophage mannose receptor [48,49]. Although the catalytic and Ca^{2+}-binding loops of PLA_2s are well characterized, only a few reports provide information about the structural elements essential for the properties of PLA_2s that are independent of their phospholipase enzymic activity [32,46,50,51]. Analysis of similarities and differences in the structures of different PLA_2s, and the similarities and differences in their properties, can contribute significantly to a better understanding of various biochemical/biological processes. Based on the fact that ecarpholin S contains the amino acid residues suggested to be important for the myotoxicity of low-M_r PLA_2s [32], it can be predicted that ecarpholin S is myotoxic. Furthermore, the strong sequence identity with ammodytin L, Myo-II and App-K49, non-Asp-49 PLA_2s that represent the first group of proteins shown to be able to undergo acylation by spontaneous reaction with non-esterified fatty acids [52], predicts that ecarpholin S is capable of autocatalytic acylation.

In summary, the results of the present study show that Asp-49 is not an absolute prerequisite for the enzymic activity of low-M_r PLA_2s. Ecarpholin S, a Ser-49 PLA_2, is enzymically active on phospholipid micelles, and also induces Ca^{2+}-dependent platelet aggregation. Ecarpholin S may represent the second member of a distinct class of PLA_2s, the Ser-49 PLA_2s, which may have unique characteristics compared with Asp-49 and Lys-49 PLA_2s.

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