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Identification of Arg-30 as the Essential Residue for the Enzymatic Activity of Taiwan Cobra Phospholipase A_2[1]

Long-sen Chang,[2] Shinne-ren Lin, and Chun-chang Chang

Department of Biochemistry, Kaohsiung Medical College, Kaohsiung, Taiwan

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Taiwan cobra (*Naja naja atra*) phospholipase A_2 (PLA_2) was inactivated by arginine-specific reagents, phenylglyoxal and 1,2-cyclohexanedione. Kinetic analyses of the modification reaction revealed that the inactivation of PLA_2 followed pseudo-first-order kinetics and the loss of activity was correlated with the incorporation of one molecule of modification reagent per PLA_2 molecule. This was confirmed by the results of amino acid composition determination, that showed that a marked decrease in enzymatic activity was associated with the modification of one arginine residue. Tryptic cleavage of the modified protein and microsequencing revealed that Arg-30 was the functionally essential residue. The incorporation of a modifier into the PLA_2 did not significantly affect the secondary structure of the enzyme, as revealed by the CD spectrum, and Ca^{2+}-binding of the modified PLA_2 was unaffected. Nevertheless, the nonpolarity of the active site of PLA_2 markedly decreased with the arginine modification, as evidenced by the decreases in the enhancement of Trp and 8-anilinonaphthalene sulfonate fluorescence. These results, together with those of X-ray crystallographic analysis of *N. naja atra* PLA_2 [Scott *et al.* (1990) *Science* 250, 1541-1546], demonstrate that Arg-30 is one of the residues involved in the interfacial binding of a PLA_2 molecule with its substrate.

Key words: phospholipase A_2, modification of an arginine residue, nonpolarity of the active site, snake venom.

The enzyme, phospholipase A_2 (PLA_2, EC 3.1.1.4), is found in various species in the animal kingdom, notably in the pancreatic tissues of mammals, and the venom of snakes and bees. It specifically catalyzes the hydrolysis of the fatty acid ester bond at position 2 of 1,2-diacyl-*sn*-phosphoglycerides in the presence of Ca^{2+} (*1*).

Taiwan cobra (*Naja naja atra*) PLA_2 is an acidic single chain polypeptide consisting of 119 amino acid residues (*2*). X-ray crystallographic analysis has provided a model of its three-dimensional structure, and revealed a probable mechanism for its interfacial catalysis (*3, 4*). Scott *et al.* suggested that residues Leu-2, Tyr-3, Lys-6, Ile-9, Trp-18, Arg-30, and Tyr-63 constituted the hydrophobic channel involved in the interaction of an enzyme molecule with a phospholipid/substrate (*4*). The results of chemical modification studies were in line with this proposition, indicating that intact Tyr-3, Lys-6, Trp-18, and Tyr-63 of *N. naja atra* PLA_2 are critical for its activity (*5-8*). In contrast to the results of a number of studies on other functional side-chains, little information is available on the involvement of Arg residues in PLA_2 activity. The inactivation of porcine pancreatic PLA_2 by phenylglyoxal revealed that a single arginine was necessary for its activity (*9*), and that this arginine-specific reagent was mostly incorporated at Arg-6 (*10*). Noticeably, the residue corresponding to Arg-30 in *N. naja atra* PLA_2 is Leu-31 in porcine pancreatic PLA_2 (*1*). Although the modification of arginine residues of *N. naja atra* PLA_2 was carried out by Yang *et al.* (*11*), there have been no lines of evidence supporting the functional involvement of Arg-30 in its enzymatic activity. To resolve this problem, the modification of PLA_2 with arginine specific reagents was undertaken in the present study. Our data demonstrate, for the first time, that the integrity of Arg-30 is essential for maintenance of the nonpolarity of the active site of PLA_2, and is closely associated with the catalytic activity of PLA_2. The results are presented in this report.

MATERIALS AND METHODS

Phospholipase A_2 (PLA_2) was purified from *N. naja atra* venom according to the method previously described (*12, 13*). 1,2-Cyclohexanedione, phenylglyoxal, and 8-anilinonaphthalene sulfonate (ANS) were purchased from Sigma Chemical. SynChropak RP-P (46 mm × 25 cm) was purchased from SynChrom (Lafayette, IN, USA). All other reagents were of analytical grade.

Chemical Modification of Arginine Residues with Phenylglyoxal—One micromole of PLA_2 in 7 ml of 0.1 M sodium bicarbonate (pH 8.5) was incubated with a 12-fold molar excess of phenylglyoxal. After 20, 40, 80, and 120 min, samples were withdrawn and then desalted by passage through a Sephadex G-25 column equilibrated with 0.1 M acetic acid.

For kinetic analyses, PLA_2 (0.21 mg/ml) was incubated

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[2] To whom correspondence should be addressed. Fax: +886-7-3218309, E-mail: lschang@mail.nsysu.edu.tw

with 0.4, 0.8, 1.5, and 3 mM phenylglyoxal, respectively. At appropriate time intervals, samples were withdrawn for measurement of enzymatic activity.

Modification of Arginine Residues with 1,2-Cyclohexanedione—PLA_2 was modified with 1,2-cyclohexanedione according to the method described by Patty and Smith with a slight modification (*14*). PLA_2 (0.42 mg/ml) in 50 mM sodium borate buffer (pH 8.5) was incubated with 30, 40, 60, and 120 mM 1,2-cyclohexanedione, respectively. At appropriate time intervals, samples were withdrawn for determination of the residual activity.

Identification of the Modified Arginine Residues—To determine the positions of the arginine residues modified in the sequence of PLA_2, the phenylglyoxal-modified enzyme was reduced and *S*-carboxymethylated (RCM) according to the procedures described by Chang *et al.* (7), followed by tryptic digestion. The RCM-protein was dissolved in 0.2 M ammonium bicarbonate (pH 7.8) and then trypsin was added (40:1, w/w). Digestion was carried out at 37°C for 4 h and the hydrolysate was lyophilized. The tryptic hydrolysate was separated by HPLC on a SynChropak RP-P column (4.6 mm × 25 cm) equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of 12–50% acetonitrile for 90 min. The flow rate was 0.8 ml/min, and the effluent was monitored at 230 nm.

Native Gel Electrophoresis—Gel electrophoresis on a 7% polyacrylamide gel (pH 8.9) was carried out as described by Gabriel (*15*). Electrophoresis was performed at 25°C by applying a current of 15 mA/gel for 80 min. The gel was stained with 0.2% Coomassie Brilliant Blue R-250 and destained with 10% acetic acid containing 10% methanol.

Other Tests—PLA_2 activity determination amino acid analysis and sequence determination, fluorescence measurement and CD measurement were performed in essentially the same manners as previously described (*12, 13, 16-19*).

RESULTS AND DISCUSSION

Bifunctional ketones, *e.g.* phenylglyoxal and 1,2-cyclohexanedione, are known to specifically react with arginine residues in proteins (*20*). As shown in Fig. 1, the incubation of PLA_2 with phenylglyoxal resulted in a time- and concentration-dependent loss of enzymatic activity. Moreover, the inactivation with phenylglyoxal followed pseudo-first-order kinetics. A semilogarithmic plot of the inactivation constant (k, min^{-1}) *versus* the concentration of phenylglyoxal has a slope of 0.81, indicating that a single phenylglyoxal molecule is required to inactive the enzyme. This result could be interpreted as that only one arginine residue in PLA_2 modified by phenylglyoxal is associated with its enzymatic activity. Likewise, the loss of activity was also dependent on the concentration of 1,2-cyclohexanedione, and followed pseudo-first-order kinetics (Fig. 2). A plot of the inactivation constant (k, min^{-1}) against the concentration of 1,2-cyclohexanedione has a slope of 1.04. This again indicates that one arginine residue is essential for PLA_2 activity. The second-order rate constants for the inactivation of PLA_2 by phenylglyoxal and 1,2-cyclohexanedione were 4.10 and 0.13 $M^{-1} \cdot min^{-1}$, respectively. Hence the reactivity toward PLA_2 is much higher for phenylglyoxal than 1,2-cyclohexanedione.

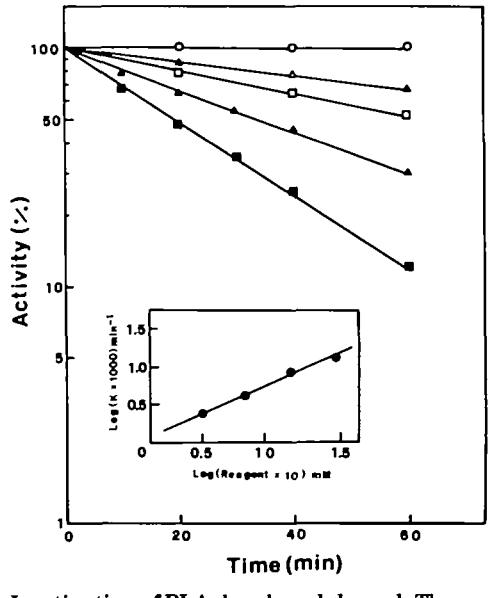

Fig. 1. **Inactivation of PLA_2 by phenylglyoxal.** The reaction was monitored as to the loss of enzymatic activity and the data are presented in a semilogarithmic manner. The modification was carried out at 25°C in 0.1 M sodium bicarbonate buffer (pH 8.5). The PLA_2 concentration in each reaction was 0.21 mg/ml. The phenylglyoxal concentrations in the various runs were: (○) 0, (△) 0.4, (□) 0.8, (▲) 1.5, and (■) 3.0 mM, respectively. Inset: Plot of the log of the pseudo-first-order rate constant, k, of the phenylglyoxal inactivation reaction *versus* the log of the phenylglyoxal concentration. The rate constants were determined from individual runs by linear least squares analysis.

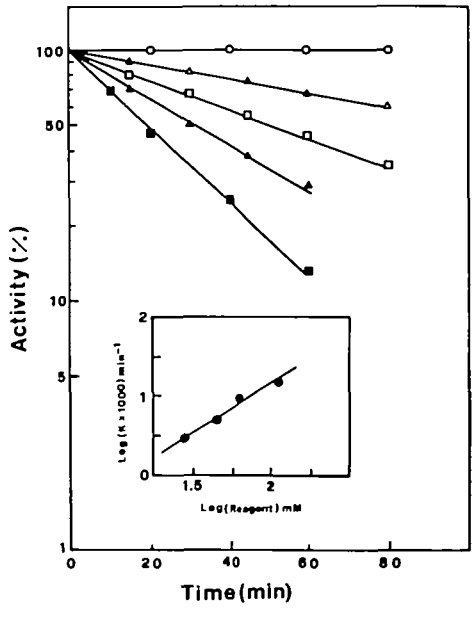

Fig. 2. **Reaction of PLA_2 with 1,2-cyclohexanedione.** The reaction was monitored as to the loss of enzymatic activity and the data are presented in a semilogarithmic manner. The modification was carried out at 25°C in 50 mM sodium borate buffer (pH 8.5). The PLA_2 concentration in each reaction was 0.42 mg/ml. The 1,2-cyclohexanedione concentrations in the various runs were: (○) 0, (△) 30, (□) 40, (▲) 60, and (■) 120 mM, respectively. Inset: Plot of the log of the pseudo-first-order rate constant, k, of the 1,2-cyclohexanedione inactivation reaction *versus* the log of the 1,2-cyclohexanedione concentration. The rate constants were determined from individual runs by linear least squares analysis.

PLA_2 was incubated with a 12-fold molar ratio of phenylglyoxal in sodium bicarbonate buffer (pH 8.5), and samples were withdrawn for analysis after 20, 40, 80, and 120 min, respectively. The enzymatic activity decreased with time (Fig. 3A). After reaction for 120 min, the residual activity was approximately 25% of the original level. The results of amino acid analysis showed that, with the exception of arginine residues, the contents of all amino acid residues remained essentially unchanged (data not shown). The data were plotted as the residual activity *versus* the number of modified arginine residues, which showed that about one arginine residue per PLA_2 molecule was correlated with the loss of PLA_2 activity (inset of Fig. 3A). As can be seen in Fig. 3B, a novel species exhibiting much slower electrophoretic mobility than the native enzyme appeared on inactivation of PLA_2 with phenylglyoxal. Meanwhile, the amount of this novel component increased with increasing reaction time. However, other modified derivatives appeared after prolonged incubation with phenylglyoxal. Since the pI values of the native and modified PLA_2 differed, as evidenced by the results of gel electrophoresis (Fig. 3B), efforts were made to separate the modified PLA_2 from the native enzyme by ion-exchange chromatography on a CM-52 or DE-52 column (Whatman International). The modified derivatives were applied on a CM-52 column (2×25 cm) equilibrated with 0.05 M ammonium acetate (pH 5.0), and eluted with a linear gradient of 350 ml of 0.05 M (pH 5.0) to 0.5 M ammonium acetate (pH 6.8) at the flow rate of 30 ml/h. Although a major peak was obtained with this purification process, both the native and modified PLA_2 were present in this fraction, as revealed by the results of gel electrophoresis (data not shown). Alternatively, the modified derivatives were applied on a DE-52 column (2×25 cm) equilibrated with 20 mM Tris (pH 8.0), and eluted with a linear gradient of 350 ml of 0 to 0.6 M NaCl in the same buffer at the flow rate of 30 ml/h. Separation of the native PLA_2 from the Arg-modified PLA_2 was also not successfully achieved. Thus, modified samples were directly analyzed without further separation. Samples were withdrawn after 20, 40, and 120 min, respectively, and then subjected to reduction and *S*-carboxymethylation, followed by tryptic digestion. The hydrolysates were separated by HPLC on a SynChropak RP-P column and the elution profiles are shown in Fig. 4. Instead of peak a observed for the hydrolysate of native PLA_2, a new peak, b, appeared for the hydrolysate of the modified PLA_2, and its size increased in parallel with a decrease in that of peak a. The results of amino acid analysis and sequence determination showed that peak a represented the peptide segment comprising positions 17-30 of PLA_2, the sequence being SWWDFADYGCYCGR. Peak b, corresponding to the sequence (SWWDFADYGCY-CGRGGSGTPVDDLDR) of PLA_2 comprising positions 17-42, contained a modified arginine residue at position 30. Thus, the peak b material consisted of the peak a material and the segment comprising positions 31-42. However, no peptide fragment representing the sequence comprising

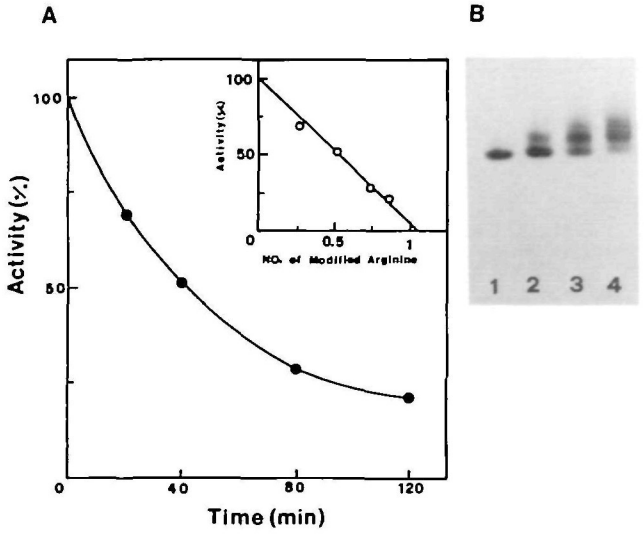

Fig. 3. **Analyses of Arg-modified PLA_2 reaction mixtures.** PLA_2 (2 mg/ml) in 0.1 M sodium bicarbonate buffer (pH 8.5) was incubated with a 12-fold molar ratio of phenylglyoxal. A: The change in the enzymatic activity as a function of the reaction time. Inset: Correlation of the inactivation of PLA_2 with the number of arginine residues modified. B: Electrophoresis analyses of the Arg-modified PLA_2. Lane 1, PLA_2; lanes 2-4, samples withdrawn from the reaction mixtures after 20, 40, and 120 min, respectively.

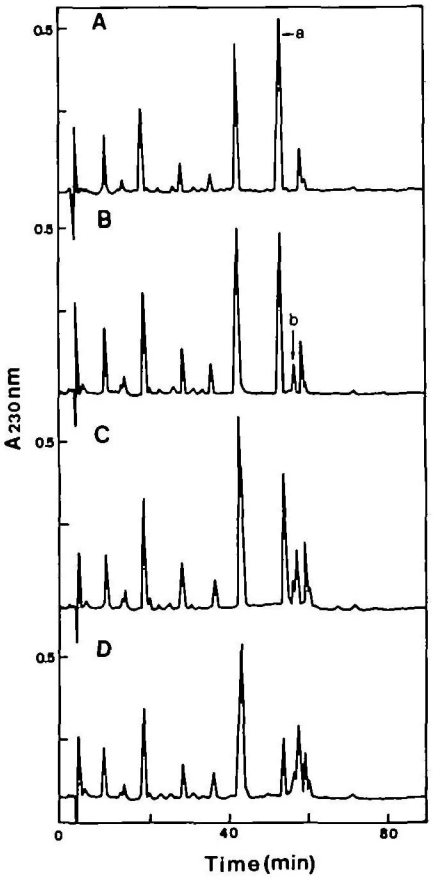

Fig. 4. **Separation of tryptic peptides from the native and modified PLA_2.** The conditions for preparation of the Arg-modified derivatives were essentially the same as given in Fig. 3. The native PLA_2 (A) and Arg-modified PLA_2 (B, 20 min; C, 40 min; D, 120 min) were subjected to reduction and *S*-carboxymethylation, followed by tryptic digestion. The tryptic hydrolysates were separated on a SynChropak RP-P column (4.6 mm×25 cm) and eluted with a linear gradient of 12-50% acetonitrile over 90 min.

positions 31-42 of PLA_2 simultaneously disappeared on tryptic peptide mapping with the inactivation reaction. Probably, this segment was co-eluted with other tryptic peptides, and thus no appreciable change was noted. Nevertheless, our results indicate that the loss of PLA_2 activity is related to the modification of Arg-30.

ANS is essentially non-fluorescent in an aqueous solution and becomes appreciably fluorescent in an apolar environment (*21, 22*). The binding of apolar ANS to PLA_2 is accompanied by enhanced fluorescence and a blue shift in the maximum emission wavelength from 520 to 460 nm (*23-25*). The binding of Ca^{2+} is associated with a further increase in fluorescence of the ANS-enzyme complex, the maximum enhancement being observed at 20 mM Ca^{2+}. The Ca^{2+}-enzyme interaction can be monitored as the change in the emission intensity of the ANS-enzyme complex caused by the addition of Ca^{2+} (inset of Fig. 5). The association constants for Ca^{2+} calculated from the lines were 1.39 and 1.1 mM, respectively. Although the Ca^{2+}-binding constant was calculated from the changes in the ANS fluorescence enhancement, the result was not significantly different from that determined from the UV difference spectra induced by the addition of Ca^{2+} (*5*). Therefore, the determination of Ca^{2+}-binding by monitoring ANS fluorescence could well reflect the binding ability of PLA_2 as to Ca^{2+}. It is evident that the binding affinity for Ca^{2+} is not impaired on modification with phenylglyoxal. Thus, the loss of biological activity was not related to Ca^{2+}-binding. However, a decrease in the ability to enhance ANS fluorescence was observed for phenylglyoxal-inactivated PLA_2. Since the hydrophobic pocket of *N. naja atra* PLA_2 for binding with ANS is its active site (*21, 22*), the decrease in the nonpolarity of the ANS-binding site may reflect that the hydrophobic character of the active site was perturbed on the modification of Arg-30. Moreover, the intensity of Trp fluorescence of PLA_2 decreased on phenylglyoxal inactivation (data not shown). In terms of the observations that the Trp residues of PLA_2 were crucial for the interaction of an enzyme molecule with its substrate and were in proximity to the active site (*7*), the decrease in the Trp fluorescence enhancement again reflected that the nonpolarity of the active site was perturbed by the modification of Arg-30. Nevertheless, the secondary structure of PLA_2 did not change significantly on modification, as evidenced by the CD spectrum of phenylglyoxal-modified PLA_2, which exhibited almost the same profile as that of the native enzyme (Fig. 6). Therefore, the observed decrease in PLA_2 activity could be exclusively attributed to the modification of Arg-30.

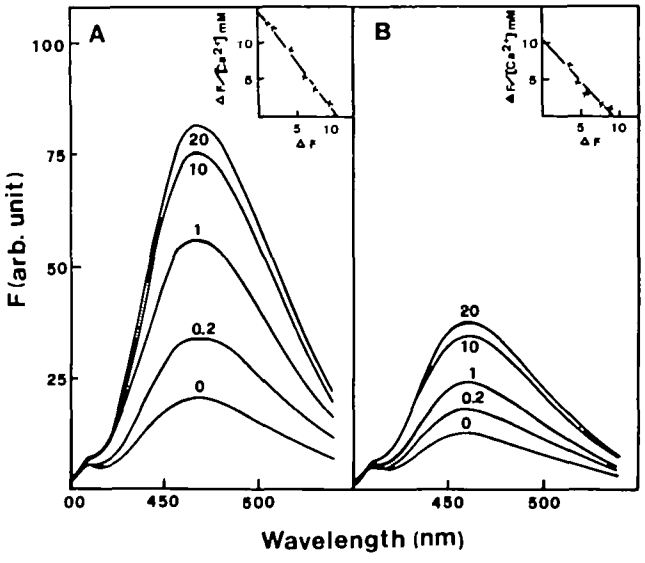

Fig. 5. **Effects of Ca^{2+} on the interaction of the native and modified PLA_2 with ANS.** The Arg-modified PLA_2 was prepared by incubation of PLA_2 with a 12-fold molar ratio of phenylglyoxal for 120 min. The sample cuvettes contained 0.105 mg of (A) native PLA_2 or (B) modified PLA_2 per ml of 0.025 M Tris-0.1 M NaCl (pH 8.0) and 7.5 μM ANS in the presence of various concentrations of Ca^{2+} (mM), as indicated. Inset: A plot of the intensity of fluorescence, ΔF, *versus* $\Delta F/[Ca^{2+}]$ gives a line with a slope corresponding to the association constant of the enzyme molecule for Ca^{2+}. The straight lines were determined by the linear regression method. Since the concentration of Ca^{2+} present was in a great excess over that of the enzyme molecule, the enzyme-bound Ca^{2+} concentration is neglected. Thus, the free $[Ca^{2+}]$ is assumed to be the initial concentration of Ca^{2+}.

The modification of Arg residues in *N. naja atra* PLA_2 had been carried out previously (*11*), and the results showed that Arg-16 and Arg-117 were the modified residues. The discrepancy between their results and ours probably arose from the reaction buffer conditions being different. In fact, a significant buffer effect on the Arg-modification reaction had been reported (*20*). In the present study, it was found that the inactivation of PLA_2 by phenylglyoxal is correlated with the extent of modification of Arg-30, showing that Arg-30 is essential for the activity of PLA_2. Although the gross conformation of PLA_2 is not drastically affected by modification with phenylglyoxal, as revealed by the CD spectrum, the decrease in the nonpolarity of active site is notable. This implies that Arg-30 should be situated in the vicinity of the active site. This is supported by the suggestion that Arg-30 is one of the residues constituting the hydrophobic channel for facilitated diffusion of a substrate/phospholipid into the catalytic site of PLA_2 (*3, 4*). Comparative analyses of the sequences

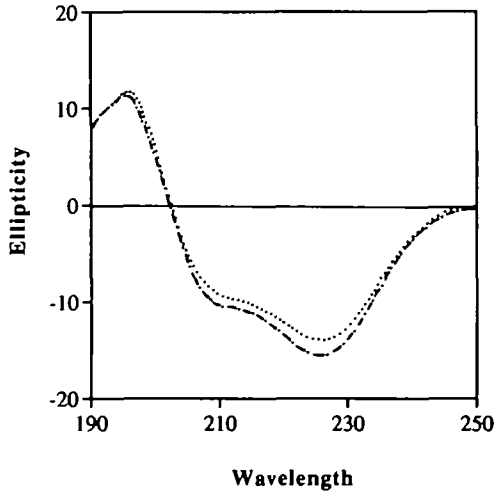

Fig. 6. **CD spectra of the native and modified PLA_2.** ····· and —·— represent the native PLA_2 and Arg-modified PLA_2, respectively. The Arg-modified PLA_2 was prepared by incubation of PLA_2 with a 12-fold molar ratio of phenylglyoxal for 120 min as described under "MATERIALS AND METHODS."

of PLA_2 enzymes revealed that the residue located at the homologous position to Arg-30 is a positively charged residue (Arg or Lys) or a hydrophobic residue (*1*). In particular, Arg-30 is highly conserved in PLA_2 enzymes from *Naja* species. Based on these observations, it is likely that the functional involvement of Arg-30 in PLA_2 activity is associated with its aliphatic moiety rather than its guanidino group. This proposition was supported by the finding that the substitution of Leu-31 of porcine pancreatic PLA_2 (which corresponds to Arg-30 of *N. naja atra* PLA_2) with Trp or Phe by site-directed mutagenesis notably increased its affinity for a monomeric substrate (*26*). Thus, it is conceivable that modification of Arg-30 might directly perturb the hydrophobic channel of PLA_2, and thereby cause a loss of enzymatic activity.

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REFERENCES

1. Waite, M. (1987) *Phospholipase*, pp. 155-241, Plenum Press, New York
2. Pan, F.M., Yeh, M.S., Chang, W.C., Hung, C.C., and Chiou, S.H. (1994) Sequence analysis and expression of phospholipase A_2 from Taiwan cobra. *Biochem. Biophys. Res. Commun.* **199**, 969-976
3. White, S.P., Scott, D.L., Otwinowski, Z., Gelb, M.H., and Sigler, P.B. (1990) Crystal structure of cobra venom phospholipase A_2 in a complex with a transition state analogue. *Science* **250**, 1560-1563
4. Scott, D.L., White, S.P., Otwinowski, Z., Yuan, W., Gelbs, M.H., and Sigler, P.B. (1990) Interfacial catalysis: The mechanism of phospholipase A_2. *Science* **250**, 1541-1546
5. Yang, C.C., Huang, C.S., and Lee, H.J. (1985) Studies on the status of tyrosyl residues in phospholipase A_2 from *Naja naja atra* and *Naja nigricollis* snake venoms. *J. Protein Chem.* **4**, 87-102
6. Yang, C.C. and Chang, L.S. (1989) Studies on the status of lysine residues in phospholipase A_2 from *Naja naja atra* (Taiwan cobra) snake venom. *Biochem. J.* **262**, 855-860
7. Chang, L.S., Kuo, K.W., and Chang, C.C. (1993) Identification of functional involvement of tryptophan residues in phospholipase A_2 from *Naja naja atra* (Taiwan cobra) snake venom. *Biochim. Biophys. Acta* **1202**, 216-220
8. Chang, L.S., Hung, J.J., Lin, S.R., and Chang, C.C. (1994) Chemical modification of Lys-6 in Taiwan cobra phospholipase A_2 with 4-chloro-3,5-dinitrobenzoate. *Biochem. Mol. Biol. Int.* **33**, 1207-1213
9. Vensel, L.A. and Kantrowitz, E.R. (1980) An essential arginine residue in porcine phospholipase A_2. *J. Biol. Chem.* **255**, 7306-7310
10. Fleer, D.A.M., Puijk, W.C., Slotboom, A.J., and de Haas, G.H. (1981) Modification of arginine residues in porcine pancreatic phospholipase A_2. *Eur. J. Biochem.* **116**, 277-284
11. Yang, C.C., Yuo, T.S., and Chen, C.Y. (1997) Studies on the status of arginine in phospholipase A_2 from *Naja naja atra* (Taiwan cobra) snake venom in *Techniques in Protein Chemistry* (Marshak, D.R., ed.) Vol. VIII, pp. 267-275, Academic Press, New York
12. Chang, L.S., Lin, S.R., and Chang, C.C. (1997) Probing calcium ion-induced conformational changes of Taiwan cobra phospholipase A_2 by trinitrophenylation of lysine residues. *J. Protein Chem.* **16**, 51-57
13. Chang, L.S., Lin, S.R., and Chang, C.C. (1997) The structural variations of ε-amino groups in phospholipase A_2 enzymes from *Naja naja atra* and *Bungarus multicinctus* venoms. *J. Protein Chem.* **16**, 133-136
14. Patty, L. and Smith, E.L. (1975) Identification of a functional arginine residue in ribonuclease A and lysozyme. *J. Biol. Chem.* **250**, 565-569
15. Gabriel, O. (1971) Analytical disc gel electrophoresis. *Methods Enzymol.* **22**, 565-578
16. Chang, L.S. (1996) Chemical modification of notexin from *Notechis scutatus scutatus* (Australian tiger snake) venom with pyridoxal-5'-phosphate. *J. Protein Chem.* **15**, 473-480
17. Chang, L.S. and Yang, C.C. (1993) Structural determinants of the intrinsic fluorescence emission in notexin and phospholipase A_2 enzymes. *J. Protein Chem.* **12**, 579-583
18. Chang, L.S., Wu, P.F., and Chang, C.C. (1996) Expression of Taiwan banded krait phospholipase A_2 in *Escherichia coli*, a fully active enzyme generated by hydrolyzing with aminopeptidase. *Biochem. Biophys. Res. Commun.* **225**, 990-996
19. Chang, L.S., Chang, C.C., and Wu, P.F. (1997) The structural and functional essentiality of the N-terminal α-helix in the phospholipase A_2 of the Taiwan banded krait. *Biochem. Mol. Biol. Int.* **41**, 1247-1253
20. Lundblad, R.L. and Noyes, C.M. (1984) The modification of arginine in *Chemical Reagents for Protein Modification*, Vol. II, pp. 1-46, CRC Press, Florida.
21. Chang, L.S., Wen, E.Y., and Chang, C.C. (1996) The essentiality of His-47 and the N-terminal region for the binding of 8-anilinonaphthalene-1-sulfonate with Taiwan cobra phospholipase A_2. *J. Protein Chem.* **15**, 255-260
22. Chang, L.S., Wen, E.Y., and Chang, C.C. (1996) The structural elements of phospholipase A_2 affecting the enhancement of 8-anilinonaphthalene-1-sulfonate fluorescence. *Biochem. Mol. Biol. Int.* **38**, 617-623
23. Chang, L.S., Lin, S.R., and Chang, C.C. (1996) The essentiality of calcium ion in the enzymatic activity of Taiwan cobra phospholipase A_2. *J. Protein Chem.* **15**, 701-707
24. Chang, L.S., Chou, L., Lin, S.R., and Chang, C.C. (1996) The interaction of 8-anilinonaphthalene-1-sulfonate with His-47 of Taiwan cobra phospholipase A_2 perturbing by the binding of calcium ion. *Biochem. Mol. Biol. Int.* **39**, 335-342
25. Chang, L.S. and Lin, S.R. (1996) Modification of tyrosine-3(63) and lysine-6 of Taiwan cobra phospholipase A_2 affecting its ability to enhance 8-anilinonaphthalene-1-sulfonate fluorescence. *Biochem. Mol. Biol. Int.* **40**, 235-241
26. Thunissen, M.M.G.M., Eiso, A.B., Kalk, K.H., Drenth, J., Dijkstra, B.W., Kuipers, O.P., Dijkman, R., de Haas, G.H., and Verheij, H.M. (1990) X-ray structure of phospholipase A_2 complexed with a substrate-derived inhibitor. *Nature* **347**, 689-691