Refolding of *Trimeresurus flavoviridis* Phospholipases A_2

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Trimeresurus flavoviridis (Habu snake) venom contains phospholipase A_2 (PLA_2) isozymes which are rich in disulfide bond and show diverse activities in spite of their highly homologous structures. When reduced form of Tyr(NO_2)-67-PLA_2, which is a mimic of Asp-49-PLA_2 mutant, was oxidized at pH 8.0 in the presence of 5 mM L-cysteine and 5 mM Ca^{2+}, native form of Tyr(NO_2)-67-PLA_2 was produced as being judged from both activity regeneration and adequate HPLC profile. Reduced forms of two Lys-49-PLA_2 isozymes with extremely low lipolytic activity, which are called basic proteins I and II, also generated native forms of proteins when oxidized under the same conditions as above. Protein disulfide isomerase accelerated proper folding of reduced Tyr(NO_2)-67-PLA_2 at an initial phase of oxidation and was effective for rearrangement of incorrectly paired disulfide bonds of Asp-49-PLA_2 to native construction. However, this isomerase failed to convert reduced form of partially active L-fragment, Asp-49-PLA_2 lacking N-terminal octapeptide, to the native form, indicating that the entire sequence of protein in a nascent state is primarily required for proper folding. The present data together with the fact that reduced Asp-49-PLA_2 folded properly (S. Tanaka et al. *J. Biochem.*, **96**, 1443 (1984)) afforded a strong basis for the structure and function study of *T. flavoviridis* PLA_2s by means of in vitro mutagenesis technique.

Phospholipase A_2 (PLA_2) [EC 3.1.1.4] catalyzes the hydrolysis of the 2-acyl ester linkage of 3-*sn*-phosphoglycerides with the requirement of Ca^{2+}. We isolated and sequenced Asp-49-PLA_2[1,2] and two Lys-49-PLA_2 called basic proteins I and II[3,4] from *Trimeresurus flavoviridis* (Habu snake, Crotalidae) venom. Although basic proteins I and II are lipolytically only 1.5—1.7% as active as Asp-49-PLA_2,[3,4] they were 10 times as active as Asp-49-PLA_2 in terms of myotoxic activity (unpublished). Since these *T. flavoviridis* PLA_2 isozymes are structurally homologous as shown in Fig. 1, they might provide a powerful system for understanding the structure-function relationships of PLA_2s by means of in vitro mutagenesis technique.

Trimeresurus flavoviridis PLA_2s have high content of disulfide bond, 7 disulfide bonds for 122 amino acid residues (Fig. 1). Thus, proper folding of reduced native and mutant proteins is of primary requisite for the mutation study. In the previous work, it has been confirmed that reduced Asp-49-PLA_2 can be properly refolded under the conditions employed.[5] However, no proper folding was found for reduced form of L-fragment which is Asp-49-PLA_2 lacking N-terminal octapeptide and can be obtained by cyanogen bromide cleavage of Asp-49-PLA_2.[6] In the work reported here, we tested unfolding and refolding reversibility of Asp-49-PLA_2 with Tyr-67 nitrated, designated Tyr(NO_2)-67-PLA_2,[7,8] as a mimic of Asp-49-PLA_2 mutant and basic proteins I and II. It is known that protein disulfide isomerase [EC 5.3.4.1] is effective in acceleration of folding of reduced proteins[9—11] and in rearrangement of mis-matched disulfide bonds.[12—14] However, this enzyme has not been employed for the folding experiment of disulfide bond-rich proteins such as phospholipase A_2. Therefore, protein disulfide isomerase was tested for refolding of reduced Tyr(NO_2)-67-PLA_2 and L-fragment as well as for rearrangement of randomly cross-linked Asp-49-PLA_2.

Results and Discussion

A mononitrated Asp-49-PLA_2, Tyr(NO_2)-67-PLA_2, is 70% as active as Asp-49-PLA_2 and has the same binding affinity to Ca^{2+} Asp-49-PLA_2.[7] Tyrosine-67 is located in the site remote from the active site but its environmental polarity is affected by the binding of the ligands to the active site. In the present study, this modified enzyme was selected as a mimic of Asp-49-PLA_2

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                  1         10         20         30         40         50
Asp-49-PLA2       GLWQFENMIIKVVKK-SGILSYSAYGCYCGWGGRGKPKDATDRCCFVHDCCYGKVTGC
Basic protein I   SLVQLWKMIFQETGK-EAAKNYGLYGCNCGVGRRGKPKDATDSCCYVHKCCYKKVTGC
Basic protein II  SLVQLWKMIFQETGK-EAAKNYGLYGCNCGVGRRGKPKDATDSCCYVHKCCYKKVTGC

          60         70         80         90         100        110        120
          NPKLGKYTYSWNNGDIVCEGDG-PC-KEVCECDRAAAICFRDNLDTYDRNKYWRYPASNCQEDSEPC
          DPKMDSYSYSWKNKAIVCGEKNPPCLKQVCECDKAVAICLRENLGTYNKK-YTIYPKPFCKK-ADTC
          NPKMDSYSYSWKNKAIVCGEKNPPCLKQVCECDKAVAICLRENLGTYNKK-YTIYPKPFCKK-ADTC
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Fig. 1. Amino acid sequences of Asp-49-PLA_2, basic proteins I and II from *T. flavoviridis* venom. The alignment was made by taking into consideration of the sequences of other PLA_2s.

mutant. Figure 2 shows a time course of appearance of enzyme activity when completely reduced Tyr(NO_2)-67-PLA_2 was oxidized at pH 8.0 in the presence of 5 mM L-cysteine, 5 mM Ca^{2+}, and 0.9 M guanidine hydrochloride (1 M=1 mol dm^{-3}). The presence of 0.9 M guanidine hydrochloride was required for preventing precipitation of the reduced protein. The activity was efficiently recovered as has been observed for oxidation of reduced Asp-49-PLA_2.[5] The progress of oxidation was also monitored by HPLC (Fig. 3). Initial broad peak of the denatured protein became sharper and a sample oxidized for 7.5 h was eluted at the same retention time as that for native Tyr(NO_2)-67-PLA_2. This process appeared to be well in accord with that of the activity regeneration. Reduced Tyr(NO_2)-67-PLA_2 was oxidized in the presence of protein disulfide isomerase under the same conditions as described above. As shown in Fig. 2, the isomerase accelerated the initial regeneration rate about 2 times that in the absence of the enzyme. An oxidized sample was coeluted with native Tyr(NO_2)-67-PLA_2 on HPLC and a Mono Q column (data not shown). Protein disulfide isomerase appeared to be effective for formation of correct pairings of disulfide bonds of disulfide bond-rich protein especially in an early stage of oxidation.

Basic proteins I and II are 55% homologous in sequence to Asp-49-PLA_2 and the positions of 14 half-

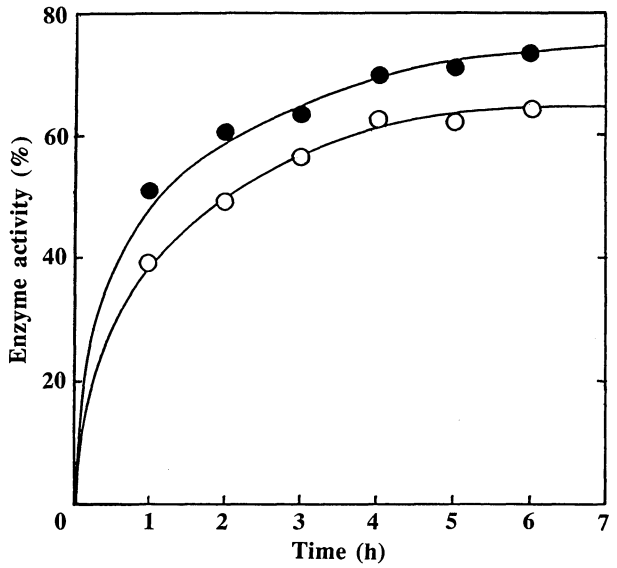

Fig. 2. Time courses of regeneration of activity when reduced Tyr-67-PLA_2 (0.05 mg ml^{-1}) was oxidized in the absence (○) and presence (●) of protein disulfide isomerase (1.5×10^{-6} M) at pH 8.0. The oxidizing solution contained 5 mM L-cysteine, 5 mM $CaCl_2$, and 0.9 M guanidine hydrochloride.

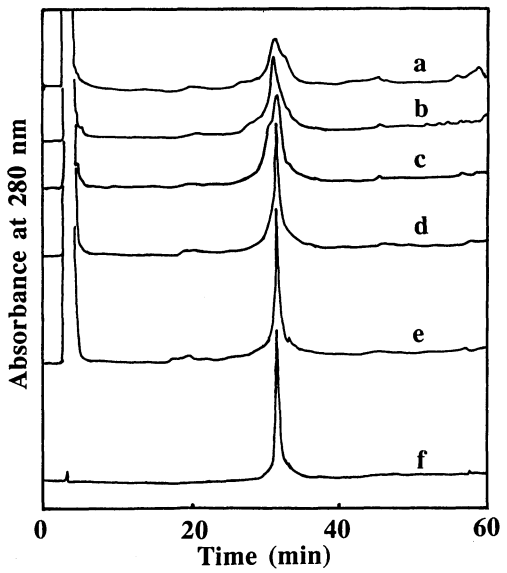

Fig. 3. HPLC profiles representing proper folding of reduced Tyr-67-PLA_2. Reduced Tyr-67-PLA_2 was oxidized under the conditions described in Fig. 2. At intervals a 200 μl aliquot was withdrawn and subjected to HPLC: TSKgel-ODS-120T column (0.46×25 cm); solvent, 0.1% trifluoroacetic acid (A)-80% acetonitrile containing 20% A (B). Elution was done with a linear concentration gradient of B, 30→100% for 60 min at a flow rate of 1.0 ml min^{-1}. Time of oxidation: a, 0 min; b, 60 min; c, 150 min; d, 450 min; e, 720 min. f, native Tyr-67-PLA_2.

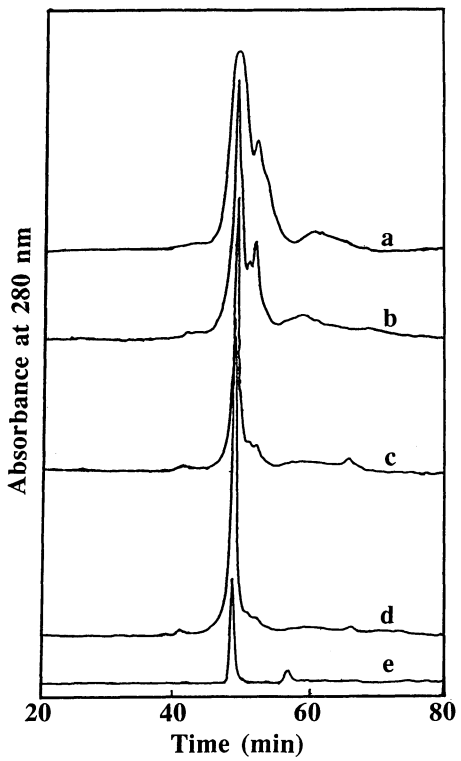

Fig. 4. HPLC profiles showing proper folding of reduced basic protein I. Oxidation of reduced basic protein I was conducted as described in Fig. 2. At intervals a 200 μl aliquot was withdrawn and subjected to HPLC as indicated in Fig. 3. Elution was done with a linear concentration gradient of B, 30→60% for 80 min at a flow rate of 1.0 ml min^{-1}. Time of oxidation: a, 0 min; b, 120 min; c, 240 min; d, 330 min; e, native basic protein I.

cystine residues are identical to those of Asp-49-PLA_2 (Fig. 1). It could be thus assumed that the tertiary structures of basic proteins I and II are greatly similar to that of Asp-49-PLA_2. Their extremely low lipolytic activity is due probably to replacement(s) of catalytically crucial amino acid residue(s) of highly active Asp-49-PLA_2. For example, Asp-49 in Asp-49-PLA_2 which has been assumed to be responsible for the binding of Ca^{2+} [15,16] is replaced by lysine in basic proteins I and II (Fig. 1). These low active PLA_2s are of invaluable counterparts of Asp-49-PLA_2 for understanding the structure-function relationships of PLA_2s through the mutation study. In this rationale, proper refolding of reduced basic proteins I and II is a matter of importance. Completely reduced basic protein I was oxidized at pH 8.0 in the presence of 5 mM L-cysteine and 5 mM Ca^{2+}. Oxidation was monitored by HPLC (Fig. 4). Initial broad peak of denatured basic protein I became sharper and a sample oxidized for 7 h was coeluted with native basic protein I. Detection of occurrence of proper folding based on enzyme activity was of difficulty because of extremely low lipolytic activity of basic protein I. Therefore, the peptide map was constructed. An oxidized sample from HPLC fractionation was digested with *Achromobacter* protease I and the digest was subjected to HPLC. The elution profile was compared with that produced by the digest of native basic protein I with the same protease (Fig. 5). Both digests gave the identical chromatographic pattern, indicating that the disulfide bond pairings in the oxidized protein are identical to those in native basic protein I. The same experiments were conducted for basic protein II. The occurrence of proper folding of reduced protein was also observed for this protein (data not shown).

It was observed in the previous work that when reduced L-fragment was oxidized in the presence of 5 mM L-cysteine and 5 mM Ca^{2+}, no proper folding occurred.[5] Although L-fragment is a protein lacking N-terminal octapeptide from Asp-49-PLA_2, it is 14% as active as Asp-49-PLA_2.[17] Thus, an assumption was made that protein disulfide isomerase may be effective for proper folding of reduced form of active protein. For the reason, oxidation of reduced L-fragment was carried out in the presence of protein disulfide isomerase under the same conditions as above. However, initial

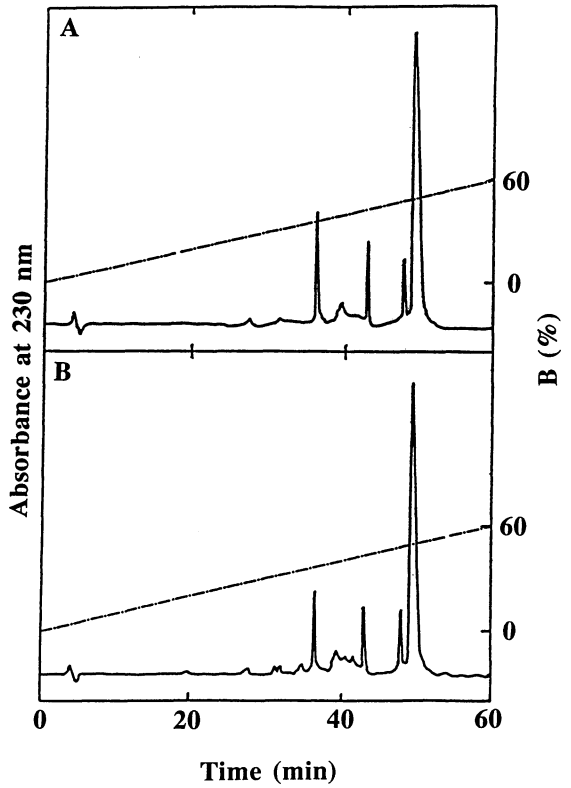

Fig. 5. Peptide mapping of native basic protein I (A) and reduced and oxidized basic protein I (B). An HPLC-purified protein (Fig. 4) was used for reduced and oxidized basic protein I. Proteins were digested with *Achromobacter* protease I as described in the text. Aliquots were subjected to HPLC as described in Fig. 3. Elution was carried out with a linear concentration gradient of B, 0→60% for 60 min at a flow rate of 1.0 ml min^{-1}.

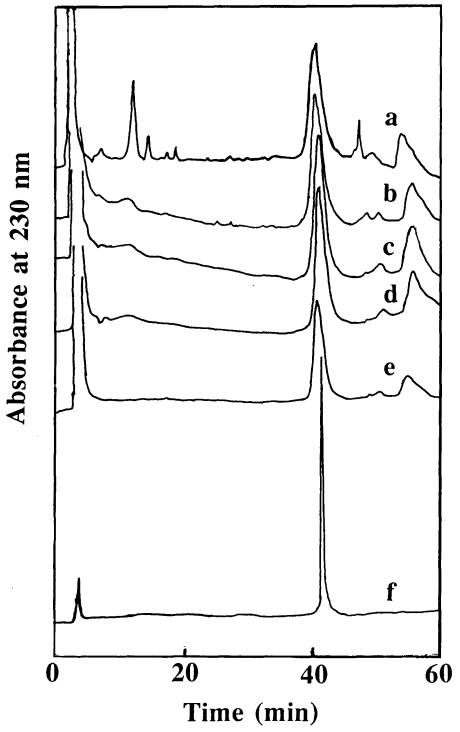

Fig. 6. HPLC profiles of reduced L-fragment oxidized in the presence of protein disulfide isomerase. Reduced L-fragment (0.05 mg ml^{-1}) was oxidized in the presence of protein disulfide isomerase (1.5×10^{-6} M) under the conditions described in Fig. 2. At intervals a 200 μl aliquot was withdrawn and subject to HPLC as indicated in Fig. 3. Elution was conducted with a linear concentration gradient of B, 0→60% for 60 min at a flow rate of 1.0 ml min^{-1}. Time of oxidation: a, 0 min; b, 30 min; c, 120 min; d, 300 min; e, 24 h. f, native L-fragment.

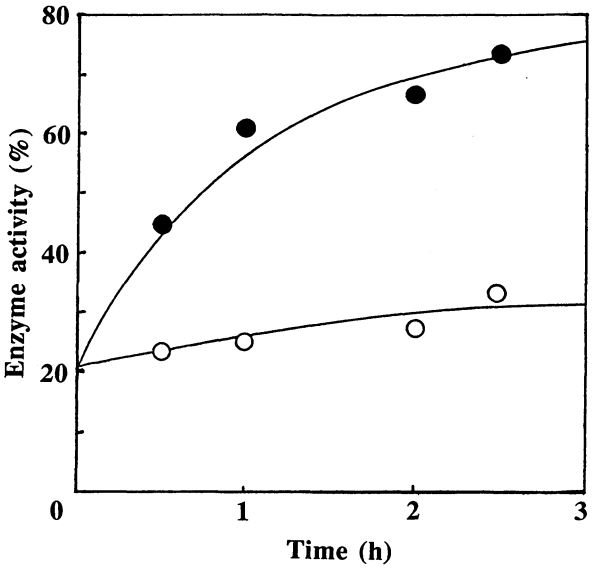

Fig. 7. Changes in activity when randomly cross-linked Asp-49-PLA_2 (0.05 mg ml^{-1}) with 20% activity was incubated with 10 mM dithiothreitol, 5 mM $CaCl_2$ and 0.9 M guanidine hydrochloride at pH 8.0 in the absence (○) and presence (●) of protein disulfide isomerase (1.5×10^{-6} M).

broad peak of reduced L-fragment remained unchanged for 24 h (Fig. 6), indicating no occurrence of correct pairings of disulfide bonds. Inefficacy of protein disulfide isomerase for proper folding of reduced L-fragment suggests that the N-terminal octapeptide sequence of Asp-49-PLA_2 is an essential component for achieving the correct pairings of the disulfide bonds and that the nascent, entire protein sequence is the definite determinant for construction of the native tertiary structure.

Randomly cross-linked Asp-49-PLA_2 with 20% activity was incubated with protein disulfide isomerase at pH 8.0 in the presence of 10 mM dithiothreitol and 5 mM Ca^{2+}. As shown in Fig. 7, activity was increased up to more than 70%. In the absence of protein disulfide isomerase, an activity increase was only to a minor extent (<5%). It became evident that protein disulfide isomerase is effective for rearrangement of incorrectly paired disulfide bonds to correct pairings even in disulfide bond-rich protein such as Asp-49-PLA_2. Protein disulfide isomerase must be useful for inducing proper rearrangement of randomly cross-linked proteins that may yield during manipulation or in the course of isolation from the cell extracts.

Establishment of unfolding and folding reversibility of Tyr(NO_2)-67-PLA_2 and basic proteins I and II together with Asp-49-PLA_2 reported previously[5] provides a sufficient basis for conducting the structure and function study of Asp-49-PLA_2 and basic proteins I and II by means of in vitro mutagenesis techniques. We have already isolated cDNAs encoding Asp-49-PLA_2,[2] basic proteins I and II, Thr-37-PLA_2, and PLX'-PLA_2[18] from *T. flavoviridis* venom gland cDNA library. Protein synthesis using these cDNAs is on the way.

Experimental

Materials. Tetranitromethane was synthesized according to the method of Liang.[19] Cyanogen bromide, dithiothreitol, and L-cysteine were purchased from Nakarai Tesque, Kyoto. Bovine pancreatic ribonuclease A and torula yeast RNA (type IX) were obtained from Sigma, St. Louis. *Achromobacter* protease I was from Wako Pure Chemical, Osaka. Other reagents were of the best grade available.

Phospholipases A_2. Asp-49-PLA_2 was purified from crude *T. flavoviridis* venom as described previously.[20] The concentration was determined by using $E^{1\%}_{1cm}$=25.6 (280 nm) and a molecular weight of 13500.[20] L-Fragment was prepared from Asp-49-PLA_2 by cyanogen bromide cleavage and purified as reported.[6] The concentration was determined using $E^{1\%}_{1cm}$=23.9 (280 nm) and a molecular weight of 12500.[6] Tyr(NO_2)-67-PLA_2 was prepared as reported.[7] Basic proteins I and II were purified as described previously.[3,4] The concentrations were determined with $E^{1\%}_{1cm}$=16.9 (280 nm) and a molecular weight of 13500. The concentrations of the reduced proteins were determined with the same extinction coefficients as those for the original proteins. Randomly cross-linked Asp-49-PLA_2 was prepared by allowing reduced Asp-49-PLA_2 to oxidize in 8 M urea. The preparation was, however, 20% active when referred to Asp-49-PLA_2.

Activity Assay. Phospholipase A_2 activity was assayed with egg-yolk emulsion and fatty acids released were titrated with 0.01 M NaOH on a Radiometer RTS-5 titration assembly consisting of a TTT2 titrator, an ABU12 autoburette, and a SBR3 titrigraph.

Protein Disulfide Isomerase. This enzyme was purified from bovine liver according to the method of Carmichael et al.[21] The concentration was determined by the method of Lowry et al.[22] with bovine serum albumin as reference and with a molecular weight of 57000.[23,24] The activity was assayed with incorrectly cross-linked ribonuclease A and with RNA as substrate for regenerated ribonuclease A.[25]

Reduction and Oxidation of Proteins. Protein (2 mg) was dissolved in 0.05 M Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride and 5 mM EDTA. After addition of 2-mercaptoethanol (5 μl), the solution was allowed to stand overnight at 25 °C. The solution was then applied onto a Sephadex G-25 column (0.7×20 cm) equilibrated with 5 mM Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride and eluted with the same buffer. The protein fractions (1 ml) were pooled and the protein concentration was determined. The solution was diluted with 0.025 M Tris-HCl (pH 8.0) to approximately 0.05 mg protein per ml and 0.9 M guanidine hydrochloride. After immediate addition of L-cysteine (5 mM) and Ca^{2+} (5 mM), the solution was allowed to stand at 25 °C. When the effect of protein disulfide isomerase was examined, the enzyme (approximately 1.5×10^{-6} M) was added together with the additives. Aliquots were withdrawn at appropriate intervals and activities were measured with reference to the same concentration of the original protein tested. Deoxygenated water was used except for buffers for oxidation.

When randomly cross-linked Asp-49-PLA_2 was treated with protein disulfide isomerase, 10 mM dithiothreitol was used instead of 5 mM L-cysteine.

HPLC. Time course of oxidation of reduced protein was

monitored by HPLC that was conducted using a TSKgel ODS-120T column (0.46×25 cm) on a Beckman liquid chromatography system consisting of two 110B solvent delivery modules, a 210A sample injection valve, a 163 UV detector, a 421A controller, and a Sic chromatocorder 11. The solvent system was 0.1% trifluoroacetic acid (A)–80% acetonitrile containing 0.02% trifluoroacetic acid (B). Elution was done with a linear concentration gradient of B.

Peptide Mapping of Proteins. Protein (1 mg/0.5 ml) was digested with *Achromobacter* protease I (10 μg) at pH 9.0 (0.02 M Tris-HCl) at 35 °C for 2 h. The digest was subjected to fractionation by HPLC.

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