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The Role of Propeptide in the Refolding of Human Group IB Phospholipase A_2

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Abstract Human group IB phospholipase A_2 (IB-PLA_2) and its zymogen (proIB-PLA_2) were purified from *E. coli*. Refolding was carried out by diluting the denatured forms of both IB-PLA_2 and proIB-PLA_2 with renaturation buffer in which the disulfide bonds were completely reduced. The refolding yield of proIB-PLA_2 was increased by about 50% over that of the mature enzyme. The refolding of IB-PLA_2 usually produced aggregates under normal conditions, as determined by light scattering. In addition, the unfolding experiments showed that the mature enzyme was more stable than the proenzyme toward denaturants in the presence of DTT. Results suggested that the N-terminal sequence rather than its conformation of human proIB-PLA_2 played an important role in the refolding process.

Key words human group IB phospholipase A_2 (IB-PLA_2); refolding; first disulfide bond; propeptide

Phospholipase A_2 (PLA_2, EC 3.1.1.4) catalyzes the hydrolysis of the 2-acyl ester bond of *sn*-3-phosphoglycerides, producing a free fatty acid and a lysophospholipid [1]. Mammalian secretory phospholipase A_2 is tentatively suggested to be involved in the inflammatory responses [2]. Human IB-PLA_2 is a member of the mammalian secretory phospholipase A_2 subgroup. Human IB-PLA_2 is first biosynthesized as a zymogen, proIB-PLA_2, and is matured by the removal of a small N-terminal peptide, Asp-Ser-Gly-Ile-Ser-Pro-Arg [3]. The propeptide suppresses IB-PLA_2 activity by two or three orders of magnitude towards its substrates as measured in the liposome form. The sequence of the human enzyme is highly homologous to those of bovine and porcine. The three dimensional structure of bovine group IB proPLA_2 has been deduced from PLA_2 [4], with the exception of two regions. One is the propeptide and the other is in the region of the 65–70 loop. These two regions display a high degree of disorder and result in an additional structural warp in residues 1–3, which are involved in the formation of the first α-helix in the mature enzyme, residues 62–64 and residue 71.

It has been reported that the propeptide always favors the refolding process, especially for those proteins containing disulfide bonds, such as bovine pancreatic trypsin inhibitor (BPTI) [5], subtilisin [6], α-lytic protease [7] and carboxypeptidase Y [8]. In BPTI, the cysteine-containing propeptide is involved in the formation of the first disulfide bond, and markedly increases the refolding yield as well as its kinetics. Propeptides, which might be longer oligopeptides or smaller peptides, have been considered as intramolecular chaperones in a wide variety of proteins.

The present investigation reports that the length rather than the conformation of the propeptide in human proIB-PLA_2 influences the refolding yield, as measured in activity by about 50% over that of the mature enzyme at 20 °C. We suggest that the propeptide might decrease the nonproductive refolding pathway and prevent the formation of aggregates.

Materials and Methods

Materials

Primers were synthesized by Bioasia Biotechnology Co., Ltd.. All restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were from TaKaRa Biotechnology Co., Ltd.. Ultra pure urea was from USB Corporation. Phospholipid β-py-C_{10}-PG [1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-

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glycero-3-phosphoglycerol] was from Molecular Probes. DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)], trypsin, bovine serum albumin (BSA, essentially fatty acid free), reduced and oxidized glutathione (GSH and GSSG) were Sigma products. All other reagents were of analytical grade.

Purification of human IB-PLA$_2$, proIB-PLA$_2$ and mutants

Human IB-PLA$_2$ and proIB-PLA$_2$ were expressed and purified as described previously [9] with a few modifications. The expressed proIB-PLA$_2$ has an additional methionine flanking the N-terminus. Plasmids for mutants were constructed using specific primers, expressed and purified in the same manner as the IB-PLA$_2$: 5′-GTG-GATCCTGCCATATGCGTGCCGTGTGGCAGTTCCGC-3′ for *mrPLA$_2$* (MRPLA$_2$), 5′-GTGGATCGTGCCATA-TGGGCATCAGCCCGCGT-3′ for *giPLA$_2$* (GISPRPLA$_2$), and 5′-GTGGATCGTGCCATATGGAAAGCGCGC-TGAGCGCGCGTGCCGTGTGG-3′ for *alphaPLA$_2$* (MESALSARPLA$_2$).

Denaturation and renaturation

The expressed protein was purified and dissolved in 50 mM Tris-HCl, pH 8.0, containing 8 M urea and 0.1 M DTT. The reaction mixture was incubated at room temperature overnight. The excess DTT was removed by passage through a desalting column (HiTrap desalting column, 5 ml, Pharmacia) equilibrated with 8 M urea, pH 3.0. The protein concentration was determined spectrophotometrically and calculated with the absorbance at 280 nm. Renaturation was performed in the refolding buffer (50 mM Tris-HCl, pH 8.0, containing 4 mM GSH and 0.8 mM GSSG) at 20 °C, except where indicated. The refolding reactions were initiated by the dilution of the unfolded protein to make a final protein concentration of 0.1 mg/ml. The enzyme activities were followed at the indicated time intervals. Aliquots were quenched by the addition of 1% trifluoroacetic acid and the free thiols were determined by Ellman's method [10]. The extent of aggregation was determined by light scattering using a Hitachi F-4010 fluorescence spectrometer. The excitation and emission wavelengths were both set at 360 nm.

Enzyme assay

The assay was carried out as reported in previous work using pyrene labeled phospholipids β-py-C$_{10}$-PG as substrates [11]. The proIB-PLA$_2$ was activated with trypsin immediately before assay using the purified IB-PLA$_2$ or proIB-PLA$_2$ as standard.

Results

The denatured and reduced IB-PLA$_2$ or proIB-PLA$_2$ in 8 M urea, pH 3.0, was diluted to 0.1 mg/ml at 20 °C with the refolding buffer (50 mM Tris-HCl, pH 8.0, 4 mM GSH and different concentrations of GSSG), and the enzyme activities were followed. No enzyme activity was detected in the absence of GSH. The addition of GSSG improved the recovery of enzyme activity. The molar ratio of GSSG/GSH was varied from 1.00 to 0.02. The optimal GSSG/GSH ratio for the refolding of IB-PLA$_2$ and proIB-PLA$_2$ was 0.20 as shown in Fig. 1.

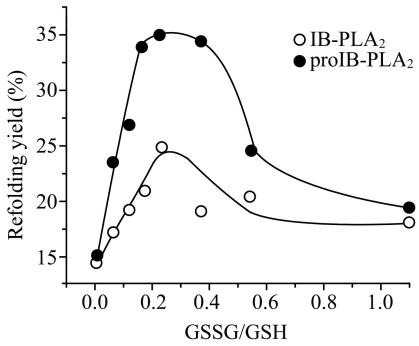

Fig. 1 Refolding yield of human proIB-PLA$_2$ and IB-PLA$_2$ at various GSH/GSSG molar ratios

0.1 mg/ml protein was refolded in refolding buffer (50 mM Tris-HCl, pH 8.0, 4 mM GSH and different concentration of GSSG) at 20 °C.

The temperature dependence of the refolding reaction of human IB-PLA$_2$ and proIB-PLA$_2$ was shown in Fig. 2. The refolding reactions were carried out at 4 °C, 20 °C and 37 °C, in the presence of 4 mM GSH and 0.8 mM GSSG. The protein concentration was 0.1 mg/ml. The yield of active enzyme was higher at the lower temperature (4 °C) than that at the higher temperature (37 °C). It took about 3 h for the refolding process for IB-PLA$_2$ to reach equilibrium at 37 °C, and 8 h at 4 °C. The proIB-PLA$_2$ refolded slightly faster than the mature enzyme under the same conditions.

The refolding yield of human proIB-PLA$_2$ was higher than that of the corresponding mature enzyme under the same conditions. For example, at 20 °C the refolding yield was about 40% for proIB-PLA$_2$, compared with 26% for the mature enzyme.

The protein concentration dependence of IB-PLA$_2$ and proIB-PLA$_2$ in refolding was shown in Fig. 3. The

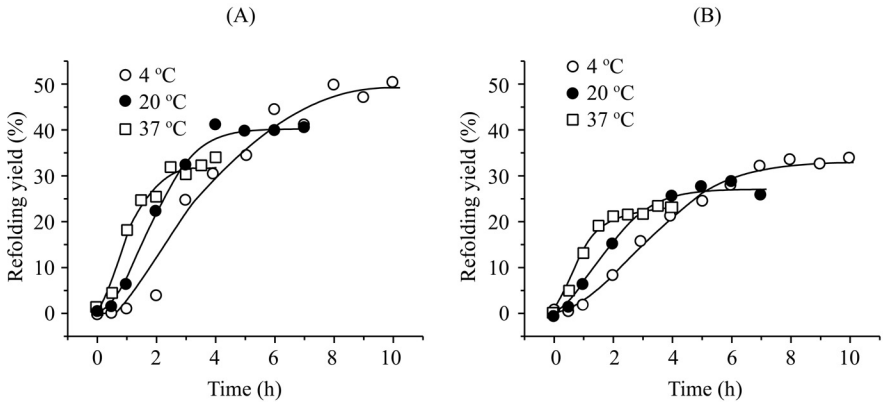

Fig. 2 Temperature dependence of human proIB-PLA_2 (A) and IB-PLA_2 (B) refolding

0.1 mg/ml protein was refolded in refolding buffer (50 mM Tris-HCl, pH 8.0, 4 mM GSH and 0.8 mM GSSG) at 4 °C, 20 °C and 37 °C, respectively.

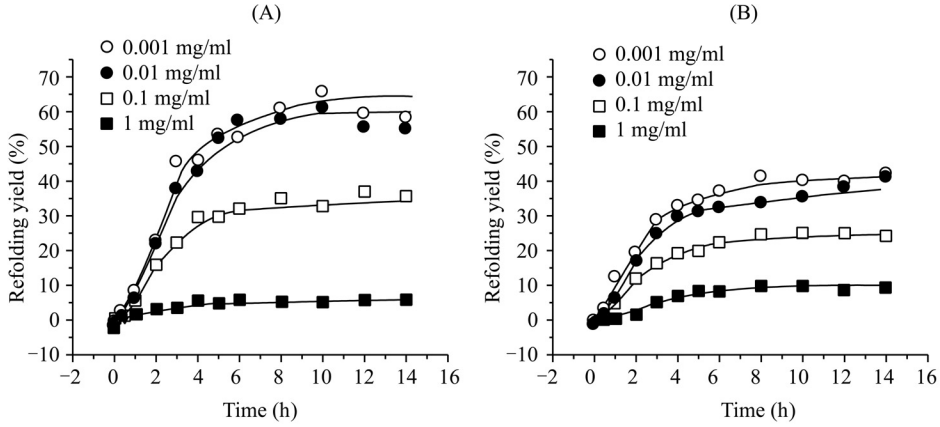

Fig. 3 Protein concentration dependence of human proIB-PLA_2 (A) and IB-PLA_2 (B) refolding

Refolding was carried out in refolding buffer (50 mM Tris-HCl, pH 8.0, 4 mM GSH and 0.8 mM GSSG) at 20 °C.

refolding yields of IB-PLA_2 and proPLA_2 were lower than 10% when the final protein concentration was higher than 1 mg/ml. The refolding yield increased strikingly by decreasing the protein concentration. The refolding yield of IB-PLA_2 reached 40% (when the final protein concentration was 1 μg/ml) and 60% for proIB-PLA_2. The refolding rate at different protein concentration was similar.

Human IB-PLA_2 contains 14 cysteine residues forming 7 disulfide bonds. As the propeptide does not contain any cysteine residue, proIB-PLA_2 and IB-PLA_2 have the same pattern of disulfide bonds. Fig. 4 showed the formation rate of disulfide bonds during refolding process. Free -SH was detected by Ellman's method. The formation rate of disulfide bonds in IB-PLA_2 was faster than that of the precursor form. The fitted curves showed that disulfide formation followed two apparent first order reactions. The rate constants for the two proteins were remarkably different in the two phases. In the first phase, the formation of disulfide bonds for IB-PLA_2 (2.3×10^{-2} min^{-1}) was about three times faster than that for proIB-PLA_2 (6.8×10^{-3} min^{-1}), and in the second phase, proIB-PLA_2 (2.9×10^{-3} min^{-1}) was faster than IB-PLA_2 (6.0×10^{-4} min^{-1}). It indicates that in the second phase, proIB-PLA_2 may still have a process for disulfide exchanges. The slow formation of disulfide bonds might be the mechanism by which proIB-PLA_2 gives rise to a high yield. This could be achieved by avoiding the formation of incorrect disulfide bonds and preventing the formation of a non-native conformation, as a consequence of decreasing the formation of aggregates.

Fig. 5 showed the formation of aggregates during

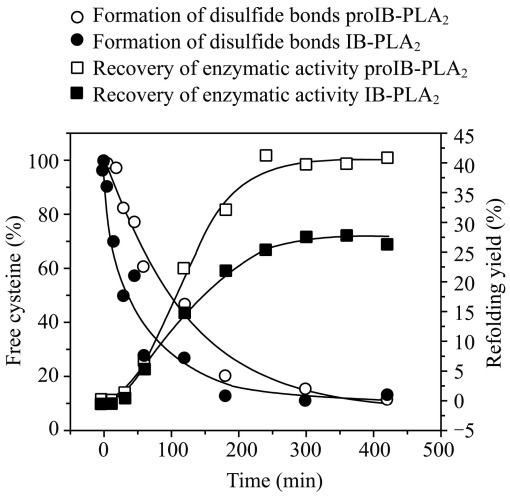

Fig. 4 Formation of disulfide bonds and recovery of enzymatic activity in human IB-PLA$_2$ and proIB-PLA$_2$ during oxidized refolding

0.1 mg/ml protein was refolded in refolding buffer (50 mM Tris-HCl, pH 8.0, 4 mM GSH and 0.8 mM GSSG) at 20 °C.

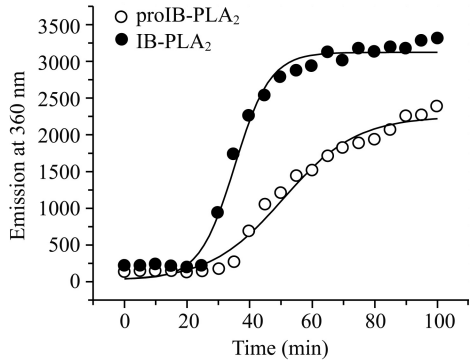

Fig. 5 Aggregation of human IB-PLA$_2$ and proIB-PLA$_2$

Denatured and reduced proteins were diluted into refolding buffer (50 mM Tris-HCl, pH 8.0, 4 mM GSH and 0.8 mM GSSG) to 0.1 mg/ml, and incubated at 20 °C.

refolding. In the first 20 min of refolding, no aggregation could be observed. Subsequent aggregation appeared suddenly, especially when a high concentration of protein was employed. IB-PLA$_2$ had an even higher tendency to form aggregates.

We constructed two propeptide deletion mutants, *mrIB-PLA$_2$* (MRPLA$_2$) and *giIB-PLA$_2$* (GISPRPLA$_2$) and compared the regeneration of enzyme activity. The refolding yields for the two mutants were shown in Table 1. The refolding yields for both of the mutants were lower than 15% even when the refolding was carried out at 4 °C at lower protein concentrations. The other mutant, *alphaIB-PLA$_2$* (MESALSARPLA$_2$) encoded a propeptide of the same length, in which the amino acids used had a high tendency to form an α-helix. The refolding yield for the product of this mutant was consistent with that of proIB-PLA$_2$ (Table 1), suggesting that the length of propeptide rather than the conformation of the propeptide was more important for the refolding yield.

Table 1 The refolding yield of proIB-PLA$_2$ and mutants

Group	Refolding yield (%)		
	37 °C	20 °C	4 °C
proIB-PLA$_2$	32	40	49
mrIB-PLA$_2$	11	13	15
giIB-PLA$_2$	9	13	14
alphaIB-PLA$_2$	28	39	48

Discussion

The human proIB-PLA$_2$ contains a propeptide of 8 amino acid residues which favors the refolding. It does not like the proteases, such as subtilisin and α-lytic protease, in which the propeptides were reported to be intramolecular chaperones and were essential for the refolding to form active enzymes. In human IB-PLA$_2$, the unfolded enzyme could be partially refolded to give a relatively low yield, however, the refolding yield for the proenzyme was increased by about 50%.

Both the reduced and the oxidized forms of sulfhydryl reagent were essential for the refolding process to obtain the active form of the proteins. This indicates that the oxidative refolding is not a spontaneous process by using molecular oxygen as an oxidant in the refolding solution. In particular, the formation of the first correct pair of disulfide bond is especially critical for the formation of the early refolding intermediate that will lead to subsequent disulfide formation and refolding. In the refolding buffer, GSSG serves as an oxidant to oxidize the thiols in the protein, and GSH serves as a reducing reagent to reduce the disulfide bonds of the protein. The ratio of GSH/GSSG determines the redox potential. It seems that the propeptide slightly shifts the redox potential to the negative side as shown in Fig. 4. There is a time lag in the curves for the

regeneration of enzyme activity for both IB-PLA_2 and proIB-PLA_2. Almost no activity was observed within the first 20 min in IB-PLA_2 when about 10% of thiols were oxidized. In the case of proIB-PLA_2, no disulfide bonds were formed during this time interval, indicating that the formation of the first disulfide bond in the protein would alter the conformation of the protein and accelerate the formation of the subsequent disulfide bonds. The formation of the first disulfide bond might be the rate-determining step. The formation of disulfide bonds fits to two first order reactions, which indicated that the formation of disulfide bonds occurred in two phases and the recovery of the enzyme activity was relatively higher in the first stage. The comparison of the rate constant indicated that the exchange of disulfide bonds to form the correct pair might occur in the oxidative refolding of proIB-PLA_2. The rate constants of proIB-PLA_2 for the two phases were compatible while those for IB-PLA_2 were in two order magnitude of difference. The slow rate constant of disulfide bonds formation in proIB-PLA_2 in the first phase might be the main factor that renders the formation of the correct disulfide pairs to maintain the protein in a native conformation and subsequently suppresses the off-pathway of refolding. It should be noted that under the optimum conditions, the off-pathway of refolding for proIB-PLA_2 that leads to the formation of aggregates, still could not be completely avoided but it could be suppressed to a minimal extent.

Mammalian pancreatic phospholipase A_2s are high conservative in primary and three-dimensional structures. The crystal structure showed that the propeptide of bovine enzyme was unstructured, in which the first three amino acid residues in the mature enzyme are involved in an α-helix. The enhancement of the propeptide in refolding yield could have two explanations. Firstly, human proIB-PLA_2 was less stable than the mature enzyme as shown by comparison of the CD changes of the two proteins in the unfolding process. There are 14 cysteine residues in IB-PLA_2 and there are 91 ways to form the first disulfide bond. A slight change in conformation might strongly affect the accessibility of cysteine residues to their counterpart cysteine residues to form a single disulfide bond. The lower stability of proIB-PLA_2 implies that the protein is in a more relaxed state; and as a consequence, it would lower the energy barriers for a cysteine to select the appropriate cysteine to correctly form a disulfide bond. The right pair of disulfide in the refolding intermediate would certainly dominate the productive pathway and decrease the formation of non-productive precipitates, as shown by light scattering.

In addition, the Barker group introduced the concept of contact order [12,13]. The nearby interaction in the protein sequence leads to a small folding free energy barrier, which plays an important role in the earlier folding process [14]. The formation of incorrect disulfide bonds would be further amplified by the exposure of hydrophobic surfaces and lead to a predominance of the off-pathways in refolding. The folding of a nascent protein might start from N-terminal region *in vivo* [15]. Our previous work also reported that in fructose-1,6-bisphosphatase, the interactions of the peptide fragments in the N-terminal sequence might affect not only the formation of the contact order sequence of the protein, but also its own conformation [16]. Results also support the proposition that the native N-terminal sequence is more important than other regions in the refolding process.

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