Investigating conformational stability of bovine pancreatic phospholipase A₂: a novel concept in evaluating the contribution of the ‘native framework’ of disulphides to the global conformational stability of proteins

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Bovine pancreatic PLA₂ (phospholipase A₂) is a 14 kDa protein whose structure is highly cross-linked by seven disulphide bonds. We investigated the structural stability of this enzyme by the method of ‘disulphide-scrambling’ with denaturants such as urea, GdmCl (guanidine hydrochloride), GdmSCN (guanidine thiocyanate) and at high temperatures in the presence of 2-mercaptoethanol (0.2 mM) as thiol initiator. Reverse-phase HPLC was used to follow denaturation. To denature 50 % of the native protein, 1.25 M GdmSCN, approx. 3 M GdmCl and higher than 8 M urea were required. Only 20 % of the protein was denatured after 2 h at 60 °C, whereas complete denaturation was seen after 2 h at 70 °C and within 30 min at 80 °C. A distinct enhancement of stability was observed when denaturation was conducted in the presence of 10 mM calcium chloride, which has not been reported previously. CD studies of GdmCl denaturation of bovine PLA₂ showed that 2.5 M GdmCl was required to denature 50 % of the protein in the presence of 0.2 mM 2-mercaptoethanol (in agreement with the HPLC analysis), whereas 6.4 M GdmCl was necessary to denature 50 % of the protein in the absence

of a thiol initiator. Conformational stability (ΔG^{water}) was estimated to be 8.7 kcal/mol (1 cal = 4.184 J) by ‘disulphide-intact’ denaturation (where ‘native’ disulphide framework was unaffected) and 2.5 kcal/mol by ‘disulphide-scrambling’ denaturation (involved breaking of native disulphides and formation of ‘non-native’ ones). The difference, $\Delta(\Delta G^{\text{water}})$, of 6.2 kcal/mol was the conformational stability contributed by the ‘native-framework’ of seven disulphides. Using bovine PLA₂ as an example, we have demonstrated a novel comparative technique, where the conformational stability study of a disulphide-containing protein, with a common denaturant, in both the presence and absence of catalytic amounts of a thiol initiator can be used as a convenient method to estimate selectively and quantitatively the actual contribution of the ‘native disulphide bond network’ towards the global conformational stability of the protein.

Key words: bovine pancreatic phospholipase A₂, calcium-induced stability, conformational stability, disulphide scrambling, protein denaturation.

INTRODUCTION

Phospholipase A₂ (PLA₂, EC 3.1.1.4) is a diverse superfamily of enzymes, which catalyse the hydrolysis of the *sn*-2 fatty acyl bond of fatty acids to liberate non-esterified fatty acid and lyso-phospholipid [1]. They are found in most living organisms and virtually in all cell types. They play a central role in cellular processes such as phospholipid digestion, metabolism, host defence, signal transduction and maintaining the composition of the cell membrane. They are involved in the defence and inflammatory response in tissue [2]. The PLA₂ superfamily is divided into nine major groups (I–IX) [3]. Groups I–III are the smallest and evolutionarily the most elementary [4]. They share many common features: all are secreted forms, have molecular masses ranging from 13 to 15 kDa, use histidine and aspartate pair for catalysis and need calcium as a cofactor for catalysis. One significant similarity shared by them is the presence of 6–8 conserved disulphide bonds, which stabilizes the structure [3]. Bovine pancreatic PLA₂ belongs to group IB, which includes the ubiquitous pancreatic PLA₂s. It is secreted into the gastrointestinal tract and is involved in the digestion of phospholipids for dietary absorption [5]. It is a small enzyme (14 kDa) and the structure is very well characterized by X-ray crystallographic studies [6,7].

Approx. 50 % of the residues have an α -helix structure, and 10 % are in antiparallel two-stranded segments of β -structure. The His⁴⁸–Asp⁹⁹ pair forms the catalytic pair with calcium as the cofactor. The most significant feature is the presence of a framework of seven strategically placed disulphide bonds. The 14 cysteine residues involved are highly conserved among more than 40 species of secreted PLA₂s [4,8,9]. These disulphide bonds interconnect key structural elements and catalytic machinery. For instance, two major helices, each possessing a catalytic residue, are connected by two disulphide bonds (Cys⁴⁴–Cys¹⁰⁵ and Cys⁵¹–Cys⁹⁸) [7]. The N-terminus involved in the H-bonding network [7], the interfacial binding site [10] and the hydrophobic channel [11] are connected to the β -wing by the disulphide bond Cys¹¹–Cys⁷⁷. The calcium-binding loop is connected to a helix by the disulphide bond Cys²⁹–Cys⁴⁵. Therefore the disulphide bond network is involved in the maintenance of the structural and functional integrity of the enzyme. Mutational analysis of each disulphide of bovine PLA₂, where one disulphide was mutated at a time, and its effect on the functional and structural stability have been reported [12]. It was found that removal of Cys¹¹–Cys⁷⁷ disulphide resulted in a large decrease in the stability, whereas the stability was decreased to a lesser extent when other disulphides were removed. Surprisingly, an increase in stability was observed

Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; GdmCl, guanidine hydrochloride; GdmSCN, guanidine thiocyanate; PLA₂, phospholipase A₂; TFA, trifluoroacetic acid.

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by the removal of Cys²⁷–Cys¹²³, which connects the C-terminus and the loop region of the protein.

In the present study, we have investigated the conformational stability of bovine pancreatic PLA₂ using the technique of disulphide scrambling by strong denaturants such as urea, GdmCl (guanidine hydrochloride), GdmSCN (guanidine thiocyanate) and high temperatures in the presence of catalytic amounts of 2-mercaptoethanol (0.2 mM). The denaturant unfolds the protein, whereas the catalytic amount of thiol agent catalyses the breakage of native disulphide bonds and formation of non-native ones according to the unfolded conformations induced by the denaturant. As calcium is an essential cofactor for this enzyme, we investigated the stability in the presence of 10 mM calcium chloride to check whether calcium binding enhances the structural stability. We also present a comparative account of the observed stability of this enzyme with other disulphide-containing proteins whose stability was studied in our laboratory by the technique of disulphide scrambling. A comparison of the conformational stability of bovine PLA₂, estimated by both 'disulphide-intact' and 'disulphide-scrambling' denaturation, was done to quantify the actual contribution provided by the seven disulphides towards the stability of this protein. We have also provided a comparative discussion of 'disulphide-intact' and 'disulphide-scrambling' conformational stability studies of other disulphide-containing proteins and have highlighted a general concept and technique that can be employed to study disulphide-containing proteins.

EXPERIMENTAL

Materials

Bovine pancreatic PLA₂ (P-8913) was obtained from Sigma. The protein was further purified by HPLC and was shown to have a purity of more than 98 %. GdmCl, GdmSCN, urea, DTT (dithiothreitol), calcium chloride and 2-mercaptoethanol were also purchased from Sigma and have a purity grade of more than 99 %.

Denaturation of bovine pancreatic PLA₂ by disulphide scrambling

Native bovine pancreatic PLA₂ (0.5 mg/ml in 0.1 M Tris/HCl buffer, pH 8.4) was treated with different concentrations of denaturants (GdmCl, GdmSCN and urea) in the presence of 0.2 mM 2-mercaptoethanol. Denaturation was performed at 23 °C for 16 h to allow the reaction to reach equilibrium. Disulphide scrambling was then quenched by mixing the samples with equal volumes of 4 % (v/v) trifluoroacetic acid (TFA), and analysed by HPLC.

Temperature-induced disulphide scrambling

Native bovine PLA₂ (0.5 mg/ml in Tris/HCl buffer, pH 8.4) was incubated at different temperatures in the presence of 0.2 mM 2-mercaptoethanol. Thermal denaturation involves inherent problems of disulphide bond destruction if performed for long periods; therefore the disulphide scrambling could not be allowed to reach equilibrium. Instead, the thermal denaturation was done in a kinetic fashion at high temperatures (60, 70 and 80 °C) for a maximum period of 2 h. Aliquots of the sample were removed at regular intervals of time, mixed with equal volumes of 4 % TFA and analysed by HPLC.

Denaturation in the presence of calcium

Disulphide scrambling of bovine pancreatic PLA₂ at different concentrations of denaturants (urea, GdmCl and GdmSCN)

at high temperatures was also studied in the presence of 10 mM CaCl₂. The conditions of denaturation were the same as mentioned previously but 10 mM CaCl₂ was present in the reaction mixture. For chemical denaturants, the scrambling was performed for 16 h, and at high temperatures it was performed in a kinetic fashion. Corresponding controls (at similar concentrations of denaturants or same high temperatures) of protein without CaCl₂ were also subjected to scrambling simultaneously for the samples with CaCl₂ for accurate comparative analysis of denaturation with and without calcium.

CD measurements of 'disulphide-intact' and 'disulphide-scrambling' denaturation

Bovine PLA₂ (0.05 mg/ml in 0.1 M Tris/HCl, pH 8.4) was incubated (for 16 h at 23 °C) with different concentrations of GdmCl (1–8 M) with and without 0.2 mM 2-mercaptoethanol. Suitable controls for each sample were also prepared without the protein and incubated simultaneously. Far-UV CD spectra of the samples and the blanks were recorded from 260 to 210 nm. Each spectrum was an average of five scans. The control spectra were subtracted from their respective sample spectra, and the ellipticities at 222 nm were noted.

Plotting of denaturation curves

The denaturation curves were drawn by plotting the percentage conversion of native bovine pancreatic PLA₂ into the disulphide-scrambled isomers versus the respective denaturant concentrations. For thermal denaturation, the kinetic denaturation curves were drawn by plotting the percentage conversion of native protein into disulphide isomers at different intervals for a given temperature. Quantitative estimation of the native and scrambled forms was done by integration of the HPLC peak areas. For the CD measurements (assuming a two-state mode of unfolding), fraction unfolded (F_u) at each concentration of GdmCl was calculated by the following relation [13] and plotted against respective GdmCl concentrations:

$$F_u = (\theta_N - \theta_i) / (\theta_N - \theta_D)$$

where θ_N and θ_D are the ellipticities at 222 nm for the native form and fully denatured form respectively and θ_i is the observed ellipticity at 222 nm for a given concentration of GdmCl.

Reduction of native and scrambled forms of PLA₂

Native protein was denatured with 4.5 M GdmCl in the presence of 0.2 mM 2-mercaptoethanol at 23 °C for 16 h to convert most of the proteins into disulphide-scrambled isomers. Scrambling was quenched by mixing with equal volumes of 4 % TFA, and the protein was separated from the denaturant by passing through an HPLC column from which the denaturant-free scrambled protein was isolated. This sample (0.1 mg/ml in Tris/HCl buffer, pH 8.4) was reduced with 0.5 mM DTT at 23 °C. Aliquots were removed at different time intervals, acidified with equal volumes of 4 % TFA and analysed by HPLC. For comparison, the native protein was also subjected to reduction simultaneously with 0.5 mM DTT under the same conditions, and the reduction was followed at regular time intervals as mentioned above.

RESULTS

The native tertiary structure of bovine pancreatic PLA₂ is maintained by seven strategically placed disulphide bonds which

Figure 1 Disulphide scrambling of bovine pancreatic PLA₂ with denaturants

Native bovine PLA₂ (0.5 mg/ml in 0.1 M Tris/HCl buffer, pH 8.4) was treated with different concentrations of urea, GdmCl and GdmSCN in the presence of 0.2 mM 2-mercaptoethanol. Samples were incubated at room temperature (23 °C) for 16 h, after which the disulphide-scrambling reaction was quenched by adding equal volumes of 4% TFA and samples were analysed by HPLC using the following conditions: solvent A was 0.1% TFA and solvent B was acetonitrile/water (9:1, v/v) containing 0.08% TFA. The gradient was 20–65% solvent B, linear for 45 min, and then returned to 20% solvent B in 20 min. The column was Zorbax 300SB-C18 for peptides and proteins, 4.6 mm × 25 cm, 5 μm, and the column temperature was 23 °C. The flow rate was 0.5 ml/min. Chromatograms of disulphide-scrambled samples at different concentrations of the three denaturants are shown. 'N', native protein; 'X', disulphide-scrambled isomers.

play a role in bringing the residues involved in catalysis into proper vicinity and linking various secondary-structural elements of the protein. On subjecting to disulphide scrambling, the number of combinations in which the 14 cysteine residues can oxidize to form disulphide bonds and the resultant disulphide-scrambled isomers is very high. To be specific, 135 135 disulphide isomers are possible. This is clearly evident from our studies.

Disulphide scrambling with denaturants

The HPLC chromatograms of the disulphide-scrambled samples of bovine PLA₂ with different concentrations of urea, GdmCl and GdmSCN are shown in Figure 1. In the presence of denaturants and catalytic amounts of 2-mercaptoethanol, the seven native disulphides break, shuffle and re-form according to the conformation induced by the denaturant. A progressive and quantitative conversion of the native protein into a very large number of isomers was observed, as expected. These isomers are eluted as a broad HPLC peak with higher retention time than the native protein. A complete conversion of the native protein into disulphide-scrambled isomers was observed with 2 M GdmSCN or 5 M GdmCl, and only 85–90% conversion with 9 M urea, indicating that GdmSCN was more potent than GdmCl, which is in turn a stronger denaturant than urea. By integration of the HPLC peak areas, the denaturation of the native PLA₂ into disulphide-scrambled isomers could be quantified accurately. The percentage conversion of native protein at different concentrations of denaturants has been depicted in Figure 3(A) (curves with filled markers). From these curves, it can be deduced that 1.25 M GdmSCN, approx. 2.5 M GdmCl and approx. 8.25 M urea were necessary to denature 50% of the native bovine PLA₂. Therefore

GdmSCN was 2.6-fold potent than GdmCl, 6.6-fold more potent than urea in denaturing PLA₂, whereas GdmCl was 2.5-fold more potent than urea. From these denaturation curves, it is evident that the commencement and completion of denaturant occurs within a narrow range of denaturant concentrations, where the non-covalent interactions are affected, resulting in the 'melting' of the tertiary structure. For bovine PLA₂, it was in the range 0.5–2 M for GdmSCN, 2–5 M for GdmCl and 7–9 M for urea.

Disulphide scrambling at high temperatures

Scrambling of bovine PLA₂ was followed kinetically at different high temperatures of 60, 70 and 80 °C. The percentage conversion of the native protein into disulphide-scrambled forms was quantified at different intervals of time and plotted in Figure 3(B) (curves with filled or solid markers). At 60 °C, the native structure was affected only to a small extent, as evident by the denaturation of 20% of native protein after 2 h. At 70 °C, a higher rate of denaturation was observed, with 50% denaturation within 30 min and complete denaturation after 2 h. Denaturation was rapid at 80 °C, with complete denaturation within 30 min.

Calcium-induced stability of bovine pancreatic PLA₂

Calcium is an essential cofactor for the catalytic activity of this enzyme [14,15]. The protein has a distinct calcium-binding loop, where Ca²⁺ is co-ordinated to peptide α-carbonyl groups of Tyr³⁰, Gly³², Gly³⁴ and the side-chain carbonyl of Asp⁴⁹. This loop is conserved among all group IB PLA₂s. Calcium is necessary for the stabilization of the transition state during catalysis [8]. To examine the possibility of enhanced stability on calcium binding, we investigated the disulphide scrambling in the presence of 10 mM CaCl₂ with different denaturants and high temperatures. A distinct enhancement of conformational stability in the presence of calcium was observed, evident by the considerable decrease in the conversion of the native protein into the disulphide-scrambled forms (Figure 2). The conversion of native protein into disulphide isomers at different concentrations of denaturants and different temperatures was quantified, and the denaturation curves in the presence of Ca²⁺ (curves with open markers in Figures 3A and 3B) were plotted along with denaturation curves in the absence of Ca²⁺ (curves with filled markers) for comparison. A distinct shift of the denaturation curves towards the right (Figure 3A) and downwards (Figure 3B) clearly indicated the enhanced stability bestowed by calcium binding. It becomes much clearer by noting that 38, 39 and 30% of native proteins were denatured with 1.5 M GdmSCN, 3.5 M GdmCl and 9 M urea respectively, in the presence of 10 mM CaCl₂, whereas in the absence of calcium, 70, 60 and 90% of native proteins were denatured with similar concentrations of the respective denaturants (Figure 2). Similarly, 58% of native protein was denatured at 70 °C with calcium after 1 h, whereas 85% was denatured in the absence of calcium. There was no noticeable difference in the denaturation rate at 80 °C in the presence and absence of calcium, as the temperature was very drastic for the presence of calcium to make any difference. These results clearly indicate that the binding of Ca²⁺ provides enhanced stability to bovine pancreatic PLA₂.

CD studies

A comparison of the GdmCl-induced 'disulphide-intact' and 'disulphide-scrambling' denaturation of bovine PLA₂ was made by CD measurements. The far-UV CD spectra at different concentrations of GdmCl both in the absence (Figure 4A) and in the presence (Figure 4B) of 0.2 mM 2-mercaptoethanol clearly show

Figure 2 Calcium-induced conformational stability

Disulphide scrambling of bovine pancreatic PLA₂ was performed at high temperatures for different concentrations of denaturants both in the absence and presence of 10 mM calcium chloride. Conditions for denaturation and HPLC analysis were the same as mentioned in the legend to Figure 1. The chromatograms after denaturation with 9 M urea, 3.5 M GdmCl and 1.5 M GdmSCN after 16 h incubation with calcium (indicated by the symbol of Ca²⁺ nearby) and without calcium are shown. HPLC chromatogram of samples denatured for 1 h at 70 °C is also shown. The percentage denaturation of the native protein under each condition is shown for easy comparison. 'N', native protein; 'X', disulphide-scrambled isomers.

the extent of denaturation by GdmCl at two different conditions. Ellipticity at 222 nm at different concentrations of GdmCl was taken as a measure of the extent of denaturation and has been plotted in Figure 4(C). The denaturation curve from the CD measurements for the disulphide scrambling (open markers) is similar to that plotted by the HPLC analysis (Figure 3A), confirming that the denaturation commences by 2 M GdmCl and is complete with 5 M GdmCl. For 'disulphide-intact' denaturation, PLA₂ denatures at much higher concentrations of GdmCl (filled markers). Denaturation begins at 5.5 M, 50% is denatured at 6.5 M, and is complete with 8 M GdmCl. The CD studies highlight the difference in stability exhibited by this protein when two different denaturation methods were employed.

Calculation of conformational stability

From the CD data, the difference in free energy, ΔG_{app} , between the folded and unfolded conformations was calculated using

$$\Delta G_{app} = -RT \ln[(F_u)/(1 - F_u)]$$

ΔG_{app} was found to vary linearly with GdmCl concentrations for both disulphide-intact and disulphide-scrambling denaturation.

Figure 3 Denaturation of bovine pancreatic PLA₂ at different concentrations of denaturants (urea, GdmCl and GdmSCN) in the absence of calcium (filled markers) and in the presence of 10 mM calcium chloride (open markers)

The conversion of the native into scrambled forms was quantified by integration of the HPLC peak areas and plotted against respective denaturant concentrations. A distinct shift of the curves towards the right in the presence of calcium indicates stability rendered by calcium binding (A). Denaturation curves for bovine PLA₂ at three different temperatures (60, 70 and 80 °C) in the absence (filled markers) and in the presence of 10 mM calcium chloride (open markers) are shown. Kinetic studies were used for understanding the mechanism of denaturation. The percentage conversion of the native protein into scrambled forms was quantified by integration of HPLC peak areas and plotted against respective time intervals. A noticeable downward shift of the curves in the presence of calcium suggests added stability by calcium (B).

Least-squares analysis was used to fit the data to the equation

$$\Delta G_{app} = \Delta G^{water} - m(\text{GdmCl})$$

where ΔG^{water} is the value of ΔG_{app} in the absence of GdmCl and m is the measure of the dependence of ΔG_{app} on GdmCl concentration. The midpoint of GdmCl denaturation curve was calculated by the equation $(\text{GdmCl})_{1/2} = [(\Delta G^{water})/m]$, since $\Delta G_{app} = 0$ at $(\text{GdmCl})_{1/2}$. Results are summarized in Table 1.

Enhanced susceptibility of 'non-native' scrambled disulphide bonds to reduction

The native disulphide bonds in a protein are assisted and reinforced by the favourable 'native' non-covalent interactions. Consequently, the non-native disulphide bonds can be expected to

Figure 4 CD measurement of disulphide-intact and disulphide-scrambling denaturation

Bovine PLA₂ (0.05 mg/ml in 0.1 M Tris/HCl, pH 8.4) was incubated (for 16 h at 23 °C) with different concentrations of GdmCl (1–8 M) with and without 0.2 mM 2-mercaptoethanol. Far-UV CD spectra of the samples and the blanks were recorded from 260 to 210 nm. The control spectra were subtracted from their respective sample spectra, and the changes in the far-UV CD spectra with every 0.5 M increment of GdmCl concentration in the absence (A) and presence (B) of 0.2 mM 2-mercaptoethanol are shown respectively. The concentrations of GdmCl at which the spectra were recorded have been indicated above the respective spectra. The units of mean residue ellipticity used are mdeg · cm² · dmol⁻¹. The spectrum labelled with 'N' represents the CD spectrum of the native protein. Decrease in the ellipticity at 222 nm was used to quantify the extent of denaturation with GdmCl by disulphide-scrambling (open markers) and disulphide-intact (filled markers) denaturation (C).

Table 1 Free energy of GdmCl-induced unfolding of bovine PLA₂

Calculated from $\Delta G_{app} = \Delta G^{H_2O} - m(GdmCl)$ at 23 °C, in 0.1 M Tris/HCl (pH 8.4) and no calcium. (GdmCl)_{1/2} is the midpoint of GdmCl denaturation curves, m the dependence of ΔG_{app} on GdmCl concentration and R the correlation coefficient. The error limit of ΔG^{H_2O} was ± 0.5 kcal/mol.

Denaturation mode	ΔG^{H_2O} (kcal/mol)	(GdmCl) _{1/2}	m	R^2
Disulphide-intact	8.7	6.4	1.34	0.965
Disulphide-scrambling	2.5	2.5	1.04	0.969

have decreased stability. We investigated the susceptibility of native and non-native disulphide bonds of bovine PLA₂ to reduction (Figure 5). The native protein and disulphide-scrambled isomers were reduced with 0.5 mM DTT and the reduction was followed kinetically. It was evident that most of the disulphide-scrambled isomers were converted into fully reduced protein 'R' within 15 min (Figure 5B). Some peaks were observed to remain (peaks a–c), which were actually partially reduced forms of the protein. On the other hand, there was a conversion of a small amount of native protein into partially reduced form 'a' after 30 min of reduction with 0.5 mM DTT (Figure 5A). This clearly indicated that the non-native disulphide bonds in the scrambled isomers were highly susceptible to reduction in comparison with the native disulphides. A similar phenomenon was observed in cases of hirudin, tick anticoagulant protein [15] and RNase A [16], where all the disulphide bonds of the scrambled isomers could be reduced with 0.5 mM DTT at room temperature within 20 min.

DISCUSSION

Disulphide scrambling is a very useful technique, which can be employed to investigate the conformational stability of disulphide-containing proteins. It involves the unfolding of the protein with denaturants in the presence of catalytic amounts of reducing agent (e.g. 0.05–0.3 mM 2-mercaptoethanol) during which the native disulphides scramble (break and re-form) according to the conformation induced by the denaturant resulting in different

Figure 5 Enhanced susceptibility of disulphide-scrambled isomers to reduction

Native bovine PLA₂ and the disulphide-scrambled isomers (0.1 mg/ml in Tris/HCl buffer) were reduced with 0.5 mM DTT at 23 °C. Aliquots were removed at regular time intervals, quenched by mixing with equal volumes of 4% TFA and analysed by HPLC. A small percentage of native protein was converted into the practically reduced form 'a', whereas most of the native protein remained intact after 30 min reduction with 0.5 mM DTT (A). Most of the disulphide-scrambled isomers were reduced to give the completely reduced form 'R' within 15 min of reduction, whereas the native protein in the sample was intact (B). The three peaks 'a–c' were partially reduced forms, resulting from the reduction of the scrambled isomers.

scrambled isomers. The extent of denaturation inflicted by the denaturant is reflected by the extent of conversion of the native protein into the disulphide-scrambled forms. This can be quantified with accuracy, as the isomers are acid-stable, and can be trapped in acid, analysed by HPLC and quantified. We have investigated the conformational stability of bovine pancreatic PLA₂ by this technique.

Table 2 Comparison of 'disulphide-intact' and 'disulphide-scrambling' denaturation

Denaturant concentrations necessary to denature 50 % of five proteins, both by disulphide-intact and -scrambling denaturation have been tabulated to highlight the difference in the necessary denaturant concentrations by the two methods.

Protein	Protein mass (amino acids)	No. of disulphides	Denaturant	Disulphide-intact	Disulphide-scrambling
α -Lactalbumin (calcium-bound)	122	4	GdmCl	3.75 M [20]	2.25 M [21]
RNase A	124	4	Urea	7.5 M [19]	6.0 M [16]
BPTI	58	3	GdmCl	Highly resistant even at 8 M [22]	7.5 M [23]
Human α -thrombin	295	4	GdmCl	0.75 M [24]	0.75 M [25]
Bovine PLA ₂	123	7	GdmCl	6.4 M (this work)	2.5 M (this work)

The HPLC chromatograms of bovine pancreatic PLA₂, subjected to disulphide scrambling with different concentrations of urea, GdmCl and GdmSCN, are shown in Figure 1. As the protein has seven disulphide bonds, which break and shuffle during denaturation, the number of different combinations in which the 14 cysteine residues can oxidize and the resultant isomers is very high (135 135 possible isomers). The conversion of the native protein into these numerous isomers was observed in our study, as these isomers were eluted as a broad HPLC peak with higher retention time than the native form. Owing to the high numbers of the isomers, their efficient separation, individual quantification and structural analysis become very difficult. The extent of denaturation of the native form could be accurately quantified and plotted (Figure 2). The concentration of denaturant required to denature 50 % of the protein was 1.25 M GdmSCN, approx. 3 M GdmCl and 8.25 M urea. The denaturation study that we performed with bovine pancreatic PLA₂ revealed two major results, which were in contradiction with the previously reported studies: first, a comparatively lower conformational stability of PLA₂ was observed, and secondly, an enhanced conformational stability of PLA₂ upon calcium binding was demonstrated in our study.

Lower conformational stability of PLA₂

'Disulphide-intact' denaturation study of bovine pancreatic PLA₂ had been performed and reported previously [12,17]. The signal used to monitor denaturation was ellipticity at 222 nm, which reflects the helicity of the protein. A high conformational stability was observed by these studies, where 6.4 M GdmCl was found necessary to denature 50 % of the native protein. This was confirmed by our CD studies of 'disulphide-intact' denaturation (Figure 3C). This was in contrast with the 'disulphide-scrambling' denaturation, where approx. 2.5 M GdmCl was sufficient to denature 50 % of the protein as estimated by both HPLC (Figure 3A) and CD measurements (Figure 4C). A higher conformational stability exhibited by the protein when denatured only by GdmCl in the absence of 2-mercaptoethanol was mainly due to the fact that the rigid disulphide network was not affected. Intact disulphides increase the conformational stability mainly by constraining the unfolded conformations of the protein and thereby decreasing their conformational entropy [18]. When the denaturation was conducted in the presence of catalytic amounts of 2-mercaptoethanol, the native disulphide framework was 'teased' to break and re-form according to the conformational changes induced by the denaturant. Once the rigid 'native-framework' of disulphides was rendered flexible, the conformational stability actually provided by the non-covalent interactions, which maintain the secondary and tertiary structure and supplemented the stability rendered by the disulphides, was better measured by the technique of disulphide scrambling.

The discrepancy observed regarding the concentration of denaturant necessary to denature bovine PLA₂ by our method of disulphide scrambling and the reported conventional 'disulphide-intact' denaturation was also observed in the cases of other disulphide-containing proteins, such as RNase A, α -lactalbumin, BPTI (bovine pancreatic trypsin inhibitor) and others (Table 2). RNase A (123 amino acids with four disulphide bonds) needed 7.5 M urea to denature 50 % of the native form when disulphide-intact protein was denatured [19], whereas by the method of disulphide scrambling, 6 M urea was enough to denature 50 % of the native protein [16]. For calcium-bound α -lactalbumin (122 amino acids with four disulphide bonds), 3.75 M GdmCl was needed to denature 50 % of the native form by disulphide-intact denaturation [20], whereas by disulphide scrambling, 2.25 M GdmCl was enough to denature 50 % of the native protein [21]. Similarly, 'disulphide-intact' denaturation studies of BPTI showed that it was highly resistant to unfolding with high temperature and concentrations of GdmCl [22], whereas 50 % denaturation could be achieved with 7.5 M GdmCl by 'disulphide-scrambling' studies [23]. Therefore the difference in the susceptibility of the protein to denaturants when denaturation is done by the conventional method (disulphide-intact) and by disulphide scrambling reflects the extent of dependence of the respective protein on the rigid framework of its native disulphide bonds to maintain its native structure. This is evident on comparing the denaturation of bovine PLA₂ with that of RNase A and α -lactalbumin. The difference in the susceptibility of RNase A and α -lactalbumin to denaturants when studied by the method of disulphide scrambling and by conventional denaturation methods was clearly noticeable, but not as drastic as observed for bovine PLA₂. These three proteins are of the same mass, but bovine PLA₂ has seven disulphides, whereas both RNase A and α -lactalbumin have four disulphides each. The maintenance of the native structure in bovine PLA₂ is dependent on the disulphide framework to a greater extent in comparison with the other two proteins, resulting in a comparatively higher susceptibility to denaturant action in the presence of 0.2 mM mercaptoethanol. This concept can be further strengthened by citing the example of α -thrombin, which has a light A-chain (36 residues) and a heavy B-chain (259 residues) linked covalently by an inter-chain disulphide. B-chain has three intra-chain disulphides. When the stability of human α -thrombin was studied by 'disulphide-intact' denaturation, where Ser²⁰⁵-labelled spin labels were monitored, it was found that half-maximal changes (50 % denaturation) were observed between 1–3 M urea and 0.4–1 M GdmCl [24]. This agreed remarkably with the 'disulphide-scrambling' studies conducted in our laboratory, where 0.75 M GdmCl and 2.5 M urea were necessary to denature 50 % of the protein [25], indicating there was no discrepancy between the two methods of denaturation. It can be interpreted that α -thrombin does not rely to a great extent on the disulphide bond network for its

stability. This may be true because of the unique arrangement of disulphides in α -thrombin, where the native disulphides in the B-chain are formed between three consecutive pairs of cysteine residues (Cys²⁸–Cys⁴⁴, Cys¹⁷³–Cys¹⁸⁷ and Cys²⁰¹–Cys²³¹) forming the smallest possible disulphide loops [26]. This arrangement does not provide a large amount of stability, as it does not cross-link distant regions of the sequence, which might have resulted in a more compact and stable structure. Therefore it is obvious that the difference in disulphide-scrambling and disulphide-intact denaturation indicates the extent of the dependence of a protein on its native disulphide network for stability.

Enhanced conformational stability of PLA₂ upon calcium binding

A distinct enhancement of stability of bovine pancreatic PLA₂ was observed in our studies when denaturation was conducted in the presence of 10 mM CaCl₂ by both denaturants and high temperatures (as evident in Figures 2 and 3). This enhanced stability was not observed in the previously reported 'disulphide-intact' denaturation studies of bovine PLA₂. Mutational analysis studies of PLA₂ in which the highly conserved Asp⁴⁹ (whose carboxylate side chain is involved in calcium ligation) was replaced with Asn, Glu, Gln, Lys and Ala have been reported previously [27]. It was found that these mutant proteins lost the capacity to bind calcium, and had no catalytic activity. However, their conformational stabilities were found to be comparable with the wild-type protein, with approx. 6.8 M GdmCl being necessary to denature 50% of the native protein [27]. Hence, it was concluded that calcium was essential for the catalytic activity, but not for conformational stability, which was contradictory to our studies. The main reason why the calcium-induced stability of bovine pancreatic PLA₂ was evident in our studies, but not in previous reports, also lies in the way the denaturation was performed. On observing the 'disulphide-scrambling' denaturation curves of GdmCl-induced unfolding (Figure 3A), in the present study, it was evident that the native protein, in the presence of calcium, begins to denature at 2.5 M GdmCl, 50% denaturation was seen at 3.75 M GdmCl, and complete denaturation at 6 M and higher concentrations. The calcium-induced stability was not enough to prevent denaturation beyond 6 M GdmCl. In the previously reported denaturation curves [12], the protein begins to denature at 6 M GdmCl, 50% denaturation is observed at 6.8 M and complete denaturation at 8 M GdmCl. So the denaturation started at high concentrations of GdmCl at which the presence of calcium made no difference (as evident by the complete denaturation at 6 M GdmCl in the presence of calcium; Figure 3A). By disulphide scrambling, the denaturation occurred at considerably lower concentrations of GdmCl, and we could observe that the presence of calcium induced extra stability to the conformation.

A similar enhancement of stability on calcium binding was reported for another calcium-binding protein, bovine α -lactalbumin, from our laboratory [21], where a 2-fold increase in the stability for GdmCl denaturation was observed in the presence of 5 mM CaCl₂. For bovine pancreatic PLA₂, the calcium-induced stability was evident, but not as drastic as for the α -lactalbumin. In summary, it can be speculated that the enhanced stability enjoyed by these proteins on calcium binding could be the incentive or the driving force, which promotes their affinity for calcium.

We have presented a comparison of the stability of bovine pancreatic PLA₂ with 12 other disulphide-containing proteins whose stability was tested by different denaturants by the technique of disulphide scrambling (Figure 6). The proteins have been arranged in the increasing order of stability for GdmCl, and the concentrations of three denaturants, namely urea, GdmCl and GdmSCN, needed to denature 50% of the protein have been shown. The pro-

Figure 6 Concentrations of denaturants required to denature 50% of 12 proteins determined by disulphide scrambling, along with that of bovine pancreatic PLA₂ for comparison

The proteins are arranged in ascending order of their stability against GdmCl. The extent of denaturation is determined by the fraction of native protein converted into scrambled isomers. A standard deviation of $\pm 5\%$ was allowed. The proteins are: 1, human α -thrombin [25]; 2, α -lactalbumin (calcium-depleted) [21]; 3, potato carboxypeptidase inhibitor [29]; 4, α -lactalbumin (calcium-bound) [21]; 5, RNase A [16]; 6, lysozyme [30]; 7, insulin-like growth factor-1 [31]; 8, bovine pancreatic PLA₂; 9, leech carboxypeptidase inhibitor [32]; 10, epidermal growth factor [33]; 11, tick anticoagulant protein [34]; 12, hirudin [35]; 13, BPTI [23].

tein with lowest stability investigated by us was human α -thrombin, which has two chains (A and B chain) held together by an inter-chain disulphide. The B-chain is stabilized by three intra-chain disulphides; 0.3 M GdmSCN, 0.75 M GdmCl and 2.5 M urea were sufficient to denature 50% of human α -thrombin [25]. The most stable small protein analysed in our laboratory was BPTI, which has three disulphide bonds and required 3.25 M GdmSCN and 7.5 M GdmCl to denature 50% of its native structure [23]. According to the stability shown by bovine pancreatic PLA₂ for GdmCl, it can be placed at position 8 in the range of proteins showed.

The most important highlight of this study was the evidence of a distinct difference in the conformational stability of bovine PLA₂ when measured by two techniques of 'disulphide-intact' and 'disulphide-scrambling' denaturation [$\Delta(\Delta G) = 6.2$ kcal/mol]. This indicated that bovine PLA₂ relied greatly on the network of seven disulphide bonds for its stability. As the only difference in the condition during 'disulphide-scrambling' denaturation was the presence of catalytic amounts of 2-mercaptoethanol (0.2 mM), which resulted in the breakage and re-formation of disulphides according to the conformation induced by the denaturant, it can be deduced that out of 8.7 kcal/mol of stability of the protein, 6.2 kcal/mol was provided by the 'entire' network of native disulphide bonds. The greater the protein relies on the disulphides for stability, the greater the discrepancy in the concentration of the denaturant necessary for denaturation by these two techniques [as discussed above by citing examples of RNase A, α -lactalbumin, BPTI and human α -thrombin (see Table 2)]. This technique of comparing the 'disulphide-intact' and 'disulphide-scrambling' denaturation, with a common denaturant, to estimate selectively the stability provided by the disulphide network has been demonstrated for the first time by this study, and we propose that it can be applied as a standard and convenient technique to study practically all disulphide-containing proteins.

Finally, it is natural to wonder at this stage regarding the choice between the two techniques of 'disulphide-intact' and 'disulphide-scrambling' denaturation to measure realistically the stability of a

disulphide-containing protein. Most of the disulphide-containing proteins are secretory. The environment, in the cell where they undergo folding, transportation and secretion is on the whole oxidative, where the ratio of reduced to oxidized glutathione (GSH/GSSG) ranged from 1:1 to 3:1, in contrast with the highly reductive environment of the cytosol where it ranged from 30:1 to 100:1 [28]. The 'disulphide-intact' denaturation seems to be more appropriate under more oxidizing conditions, but it has to be borne in mind that once these proteins enter the extracellular milieu, even the presence of catalytic amounts of a thiol can promote disulphide shuffling and may drastically alter the conformational stability as observed for bovine PLA₂ and other disulphide-containing proteins, as discussed above. We have exploited this alteration of stability by the thiol to estimate the stability provided by the 'native-framework' of disulphide bonds.

We thank the support of endowment from the Welch Foundation.

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Received 27 June 2003/23 September 2003; accepted 8 October 2003

Published as BJ Immediate Publication 8 October 2003, DOI 10.1042/BJ20030968