Phospholipase A_2 Engineering. The Roles of Disulfide Bonds in Structure, Conformational Stability, and Catalytic Function†

Hongxin Zhu,‡ Cynthia M. Dupureur,§,|| Xiaoyan Zhang,‡ and Ming-Daw Tsai*,⊥

Departments of Chemistry and Biochemistry and Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210

Received September 20, 1995⊗

ABSTRACT: Site-directed mutagenesis was used to probe the contribution of each of the seven disulfide bonds of bovine pancreatic phospholipase A_2 (PLA2, overexpressed in *Escherichia coli*) to the structure, conformational stability, and catalytic function of the enzyme. Each of the seven disulfide bonds, C11−C77, C27−C123, C29−C45, C44−C105, C51−C98, C61−C91, and C84−C96, was deleted separately by changing both cysteine (C) residues to alanine (A). The structural properties of the mutants were analyzed by 1D and 2D proton NMR, the conformational stability by guanidine hydrochloride-induced denaturation, and the catalytic property by measuring kinetic parameters toward DC_8PC (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine) micelles. The results led to the following significant findings: (i) All but one (C84A−C96A) mutants have been refolded and purified by use of the same procedure for wild-type PLA2. Thus, the disulfide bonds are generally not important to the folding pathway of PLA2. (ii) The disulfide bond C11−C77 is most important to the conformation and conformational stability of the enzyme since deletion of this disulfide bond resulted in greatly perturbed NMR properties and in a decrease of 6.2 kcal/mol in conformational stability. However, the C11A−C77A mutant displayed little change in catalytic function. (iii) The effects of deleting disulfide bonds on the catalytic function of PLA2 are small, except the disulfide bond C29−C45 which connects the calcium binding loop with the helix C. However, the conformation and conformational stability of the C29A−C45A mutant are essentially unperturbed. (iv) The calcium binding affinity of the C29A−C45A mutant was found to decrease by a factor of 10 or greater. This could be the cause for the perturbation in kinetic behavior. (v) Deletion of the C27−C123 disulfide bond caused an unexpected *increase* in the conformational stability of the enzyme by 2.4 kcal/mol. The overall results are discussed in relation to the structure−function relationship of PLA2 and the roles of disulfide bonds in protein structures.

The roles of disulfide bonds in the structure, stability, and function of proteins have always been of great interest and great importance. Understanding of such problems can lead to designing new proteins, or modifying existing proteins. Phospholipase A_2 (PLA2)[1] from bovine pancreas is a small enzyme with unusually high disulfide contents: it consists of only 123 amino acids, but is highly cross-linked with 7 disulfide bonds (the enzyme contains no free cysteines). The 14 cysteines are highly conserved among more than 40 species of secreted PLA2 (Davidson & Dennis, 1990; Heinrikson, 1991; Dennis, 1993). As depicted in Figure 1,

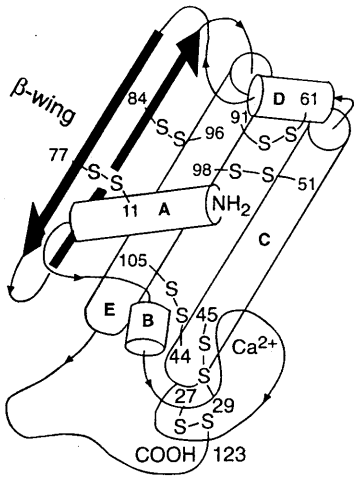

FIGURE 1: Disulfide bonds in PLA2. Reconstructed according to Dijkstra et al. (1978).

these seven disulfide bonds interconnect the key structural elements and the catalytic machineries: The two major helices of the enzyme, helix C and helix E, which contain the catalytic center, are connected by C44−C105 and C51−C98 disulfide bonds. The N-terminus, which is intimately involved in the H-bonding network (Dijkstra et al., 1981a), the interfacial binding site (Dijkstra et al., 1981b, 1984; Scott et al., 1990), and the hydrophobic channel (Scott et al., 1990), is connected to the β-wing by the C11−C77 disulfide bond. The C-terminus, which is also part of the extended interfacial

† This work was supported by Research Grant GM41788 (to M.-D.T.) from the National Institutes of Health. The Bruker AM-500 NMR spectrometer used was funded in part by NIH Grant RR01458. This is paper 15 in the series Phospholipase A_2 Engineering. For paper 14, see Liu et al. (1995).

‡ Ohio State Biochemistry Program.

§ Department of Chemistry.

|| Present address: Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77845.

⊥ Departments of Chemistry and Biochemistry and Ohio State Biochemistry Program.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: CD, circular dichroism; COSY, correlated spectroscopy; 1D, one-dimensional; 2D, two-dimensional; DC_8PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetate; GB, Gaussian broadening; GdnHCl, guanidine hydrochloride; LB, line broadening; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; pH*, pH in D_2O without correcting for the deuterium effect; PLA2, phospholipase A_2; TMSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$; WT, wild type.

0006-2960/95/0434-15307$09.00/0

binding site (Dijkstra et al., 1981b), is fixed to the main body of the protein by the C27−C123 disulfide. The calcium binding loop (residues 28−32) is connected to helix C by the disulfide bond C29−C45. The helices D and E are connected by the C61−C91 disulfide bond. The β-wing and the main helix E are connected by the C84−C96 disulfide linkage.

In this study, we report the contribution of each of the seven disulfide bonds to the structure, conformational stability, and catalytic function of bovine pancreatic PLA2 overexpressed in *Escherichia coli* (Deng et al., 1990). Each of the seven disulfide bonds was deleted by replacing both cysteine groups with alanine. The conformation of the mutants was analyzed by 1D and 2D proton NMR, the conformational stability by guanidine hydrochloride-induced denaturation, and the catalytic property by measuring kinetic parameters toward DC_8PC micelles. The results led to the following significant findings: (i) With the possible exception of C84−C96, the disulfide bonds are not critically important to the folding pathway of PLA2. (ii) The disulfide bond C11−C77 is most important to the conformation and conformational stability of the enzyme. (iii) The mutant C29A−C45A displays the greatest decrease in $k_{cat,app}$ and the greatest increase in $K_{m,app}$ toward DC_8PC micelles. The calcium binding affinity of this mutant was found to decrease by 10-fold or greater, which could be the cause for the perturbation in the kinetic behavior. (iv) The C27−C123 disulfide bond is likely to be maintaining the C-terminus at a rigid conformation suitable for the catalytic function.

MATERIALS AND METHODS

Materials and Routine Procedures. Oligonucleotides were synthesized by the Biochemical Instrument Center at the Ohio State University. Mutagenesis and sequencing kits were purchased from Bio-Rad and United States Biochemicals, respectively. DC_8PC was purchased from Avanti Polar Lipids (Birmingham, AL). Ultrapure guanidine hydrochloride was purchased from ICN Biochemicals; 99.9 atom % D D_2O, "100% D" D_2O, and TMSP-d_4 were obtained from MSD Isotopes. The Fast Flow Sepharose-S and -Q resins (cation and anion exchangers, respectively) used for FPLC columns were purchased from Pharmacia-LKB. Other chemicals and biochemicals were of the highest quality available commercially. FPLC experiments were performed on a Pharmacia-LKB FPLC system. CD spectra were recorded a JASCO J-500C spectropolarimeter using a thermostated quartz microcell.

Construction and Purification of Mutant Enzymes. For C11A−C77A and C51A−C98A, the pro-PLA2 gene from the pTO-propla2 plasmid (Deng et al., 1990) was used to construct the mutants; for C27A−C123A, C29A−C45A, C44A−C105A, C61A−C91A, and C84A−C96A, the mature PLA2 gene from the pTO-A2M plasmid (Deng et al., 1990) was used. Site-directed mutants were generated by using a Bio-Rad mutagenesis kit according to the manual provided by the manufacturer. C11A−C77A, C27A−C123A, C29A−C45A, C44A−C105A, C51A−C98A, C61A−C91A, and C84A−C96A double mutants were constructed from the oligonucleotides 5′GAAGGGATCTTAGCTTTGATCATTCC and 5′TGTTTGAAGCGCTGTAAG, 5′CCACAATAAGCACCATAATT and 5′CAAGCTTAAGCGTTTTTTTTATCAAG, 5′CCCAGGCCAGCATAACAACC and 5′TGTGTTTGGGCACAACGGTC, 5′GTTTGGCAAGCACGGTCCAG and 5′TTTGAAAAAGCAATAGCAGC, 5′TTGTTTATAGGCATTATCATG and 5′TTACGATCGGCGTTACAAA, 5′AACTTTGGCGCTATCAAGT and 5′AATGCTTCAGCTGCGTTA, and 5′CGCTGCTAGCTGTAATT and 5′CGCAGTTAGCAATAAATGC, respectively. For generating double mutants, both oligonucleotides were added to the reaction mixture simultaneously. The resulting mutants were verified by direct sequencing according to Sanger's method (Sanger et al., 1977). The recombinant PLA2 was isolated from the *E. coli* expression host BL21-(DE3) [pLysS] carrying the pTO-A2M plasmid (or the pTO-propla2 plasmid in the case of C11A−C77A and C51A−C98A mutants). Mutants with the pTO-propla2 plasmid were purified as described previously (Noel et al., 1991; Dupureur et al., 1992a,b). The purification of mutants harboring the pTO-A2M plasmid was performed by a similar procedure except that the tryptic activation step was omitted since there was no pro-sequence (Noel et al., 1991; Li & Tsai, 1993). In brief, the purification procedure consists of the following steps: (i) The inclusion body was isolated following cell breakage, detergent washing, and centrifugation. (ii) The inclusion body pellet was solubilized by sulfonation, first with sodium sulfite (which breaks a disulfide bridge into a free sulfhydryl group and a sulfonated sulfhydryl group) followed with NTSB (which sulfonates the free sulfhydryl group) (Thannhauser & Scheraga, 1985). (iii) The sulfonated protein was subjected to refolding in the presence of urea and a glutathione redox couple (de Geus et al., 1987). (iv) The refolded protein was then purified by cation-exchange chromatography (FPLC on Sepharose-S), activated by mild trypsinolysis (in the case of C11A−C77A and C51A−C98A mutants), and purified by anion-exchange chromatography (FPLC on Sepharose-Q).

NMR Analysis. Ten milligrams of enzyme (WT or mutant) was dissolved in 0.5 mL of D_2O containing 300 mM NaCl, 50 mM $CaCl_2$, kept at room temperature for 4 h to allow for deuterium exchange, and then lyophilized. After repeating the exchange step, the sample was dissolved in 0.5 mL of "100%" D_2O, and the final pH* (uncorrected pH directly from pH meter reading) was adjusted to 4.0 by using DCl and NaOD stock solutions. Spectra were obtained on a Bruker AM-500 spectrometer at 37 °C. TMSP was used as an internal chemical shift reference.

GdnHCl-Induced Denaturation and Conformational Stability. A stock solution of ca. 8.5 M GdnHCl was prepared in a buffer containing 10 mM borate and 0.1 mM EDTA, pH 8.0, and the exact concentration was determined by refractive index (Nozaki, 1972). An enzyme solution of approximately 3 mg/mL was prepared in the same buffer, and the precise concentration was measured spectrophotometrically. Typical CD samples contained, in the borate buffer mentioned above, 0.05 mg/mL enzyme and Gdn-HCl concentration varying from 0 to 8.5 M. The spectra were recorded at 30 °C with a spectral width from 200 to 250 nm and 5 scans. After subtraction of the corresponding background, the ellipticity at 222 nm was recorded and used to determine the free energy of unfolding, $\Delta G_d^{H_2O}$.

Kinetic Analysis. The activities toward micellar DC_8PC were assayed at 45 °C on a Radiometer RTSS Titration System as described previously (Noel et al., 1991). The reaction buffer consisted of a 3 mL solution containing 1 mM sodium borate, 25 mM $CaCl_2$, 0.1 mM EDTA, and 100 mM NaCl, pH 8.0. The apparent V_{max} and apparent K_m were determined from Eadie−Hofstee plots (Atkins & Nimmo,

1975) of v vs v/[S] through the use of linear regression analyses. On the basis of the molecular weight of 13 500, the apparent k_{cat} was calculated from the apparent V_{max}. For the activation study of PLA2 by Ca^{2+}, the $CaCl_2$ was omitted in the stock buffer and added, with varying concentration, to the substrate solution (5 mM DC_8PC) before the reaction.

UV Difference Spectroscopy. The affinity of the enzyme toward Ca^{2+} was measured at room temperature with a Kontron Uvikon 930 spectrophotometer interfaced with a Samsung computer. Measurements were carried out in 1-mL cuvettes at pH 8.0 (0.7 mg/mL enzyme, 50 mM Tris, 100 mM NaCl, and 0−150 mM $CaCl_2$). The ultraviolet difference spectra at 242 nm were recorded at various Ca^{2+} concentrations and used to calculate the dissociation constant (K_d) and the binding site number (n) as previously described (Pieterson et al., 1974; Tsai et al., 1985). The maximal absorption from calcium binding to the enzyme (ΔA_{max}) can be obtained from a double-reciprocal plot of absorption (ΔA) vs Ca^{2+} concentration. The saturation degree (γ) of the enzyme by Ca^{2+} equals $\Delta A/\Delta A_{max}$, and a plot of $\gamma/[Ca^{2+}]$ vs γ gives the values of K_d and n.

RESULTS

Construction and Refolding of Mutants. The specific procedures for mutant construction, expression (as inclusion bodies), refolding, and purification are described under Materials and Methods. We have found it necessary to change both cysteines of a disulfide bridge to alanine. A single Cys−Ala mutation often resulted in low yield in refolding, possibly due to mispairing of cysteines. There are precedents for the formation of non-native disulfide bonds in single cysteine mutants, as in the C81A mutant of human lysozyme (Taniyama et al., 1990). However, this is not the focus of this work, and every disulfide mutant described in this work is a double mutant. Six of the seven possible mutants, C11A−C77A, C27A−C123A, C29A−C45A, C44A−C105A, C51A−C98A, and C61A−C91A, were refolded and purified by the same procedure as used for WT PLA2. These mutants behave essentially the same as WT in every step of the purification procedure, including a cation and an anion chromatography, suggesting that these disulfide bonds are not critically important to the folding process of these six mutants.

On the other hand, the C84A−C96A mutant could not be purified even though the level of expression was similar to that of WT. Instead of a sharp peak, a very broad one was obtained from the cation-exchange column that followed the refolding step. The SDS−PAGE gel showed that the broad peak has the same molecular weight as PLA2; however, no PLA2 activity was detectable. When this sample was loaded onto the anion-exchange column used in the last step of purification, no enzyme was recovered. These results indicate that the C84A−C96A mutant protein could not be correctly refolded into a single (native) structure, and suggest that the C84−C96 disulfide bond is important to the folding process.

Structural Analysis by Proton NMR. The NMR analyses were conducted at 1.5 mM and pH* 4−5 since previous studies (Dupureur et al., 1992a,b; Li et al., 1994; Fisher et al., 1989) have shown that at higher concentration and/or neutral pH the resonances are less well resolved possibly due to microaggregation. The enzyme still maintains the native state and can be reversibly converted to neutral pH. All of our NMR studies have been performed at pH* 4−5.

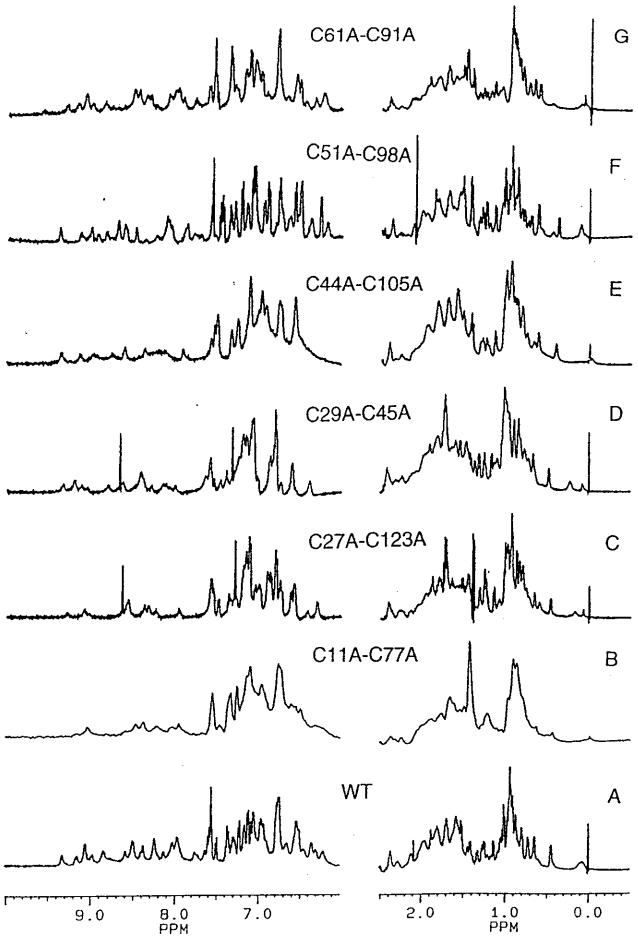

FIGURE 2: One-dimensional proton NMR spectra of WT (A), C11A−C77A (B), C27A−C123A (C), C29A−C45A (D), C44A−C105A (E), C51A−C98A (F), and C61A−C91A (G). Sample conditions: 1.5 mM PLA2, 300 mM NaCl, 50 mM $CaCl_2$, pH* 4.0, at 37 °C. All the 1D spectra except spectrum B were acquired with quadrature detection over 16k data points while spectrum B was acquired over 4K data points. After zero-filling to 32K and multiplication by a Gaussain function (LB = −5; GB = 0.1), the spectrum was obtained by Fourier transformation on an Aspect 3000 processing unit coupled to the Bruker AM-500 spectrometer.

The partial 1D proton NMR spectra of WT and the six successfully purified mutants are shown in Figure 2. Qualitatively, there are notable differences between WT and the mutants, which were further characterized by 2D NMR analyses. Figure 3 shows the NOESY spectra of the aromatic−aromatic and aromatic−aliphatic regions for WT and the mutants. It is apparent that C11A−C77A and C44A−C105A are quite distinct conformationally from WT, while the other four mutants (C27A−C123A, C29A−C45A, C51A−C98A, and C61A−C91A) display NOESY patterns very similar to that of WT. The NOESY spectrum of C11A−C77A shows the greatest perturbation among the six mutants. Very few NOE cross-peaks are observable for this mutant, which is similar to several other mutants of PLA2: H48A and H48N (Li & Tsai, 1993); and D99N, D99A, Y73S, and Y73A (Dupureur et al., 1992c). The NOESY spectrum of C44A−C105A also shows significant perturbations. Only four spin systems, L2 (Lys-58), A1 (Ala-55), M (Met-8), and Fc (Phe-94), are identifiable in this mutant; the rest of the cross-peaks have either shifted substantially or are undetectable. None of the interresidue aromatic−aromatic NOE cross-peaks that are detectable for WT can be observed in these two mutants, and there are substantially fewer interresidue aromatic−aliphatic NOE cross-peaks.

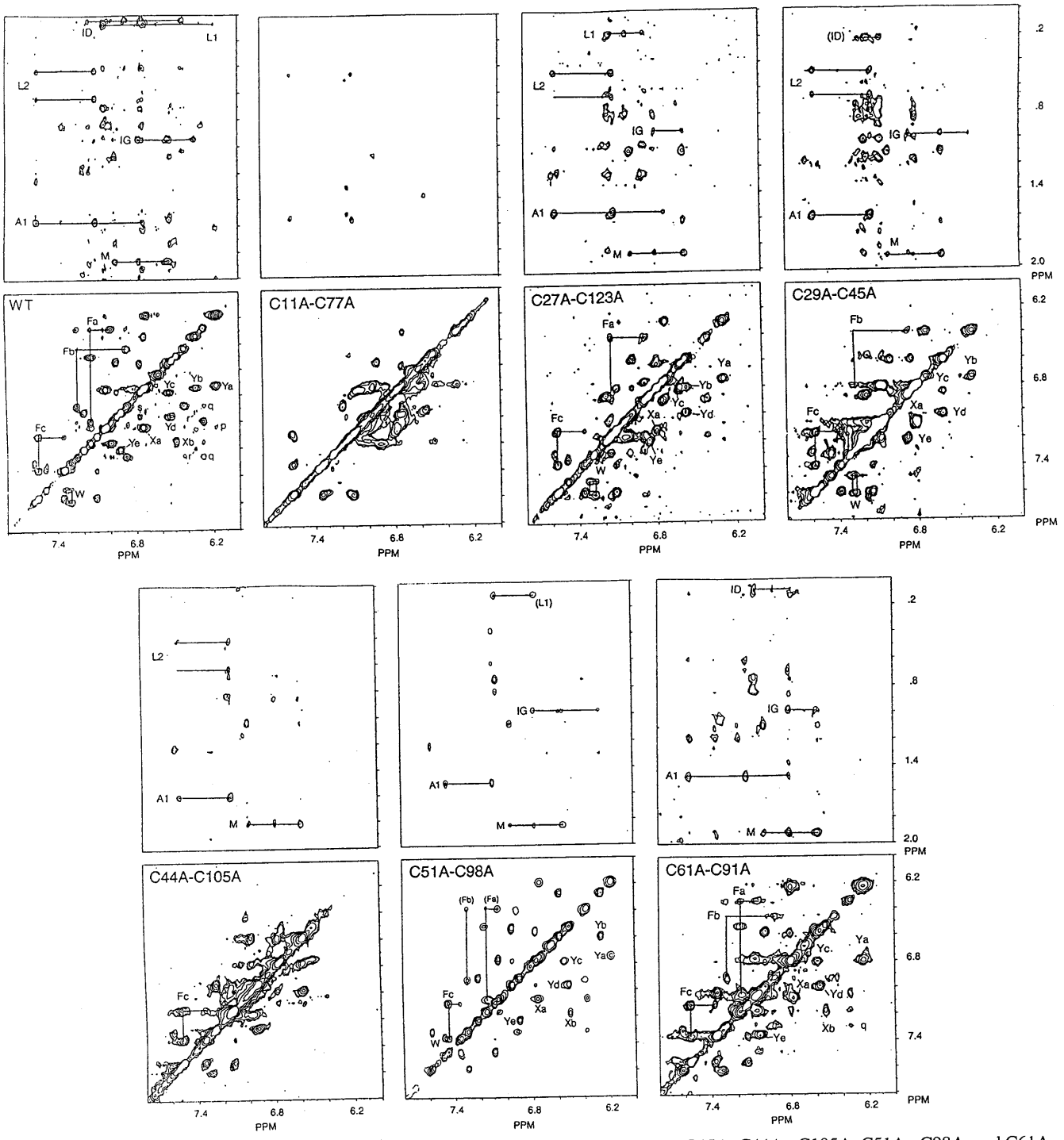

FIGURE 3: Phase-sensitive NOESY spectra of WT, C11A−C77A, C27A−C123A, C29A−C45A, C44A−C105A, C51A−C98A, and C61A−C91A in D_2O at 500 MHz. Sample conditions are the same as those described in Figure 3. The mixing time was 200 ms. A 4096 × 512 matrix in the time domain was recorded and zero-filled to a 4096 × 2048 matrix prior to multiplication by a Gaussian function (LB = −3, GB = 0.1).

The four mutants displaying smaller changes have been further analyzed semiquantitatively. On the bases of the NOESY and COSY (not shown) spectra, and the previous assignment for WT (Fisher et al., 1989; Dupureur et al., 1992a,b), the chemical shifts of most of the aromatic and some of the aliphatic resonances of C27A−C123A, C29A−C45A, C51A−C98A, and C61A−C91A have been assigned and listed in Table 1, along with the assignments for WT. There are only two resonances (Phe-94 and Tyr-52) that differ by >0.10 ppm between WT and C51A−C98A. According to the crystal structure (Dijkstra et al., 1981a,b; Noel et al., 1991), these residues are located near the mutation sites; thus, it can be concluded that the conformation of C51A−C98A is little perturbed from that of WT. The differences between WT and C27A−C123A, C29A−C45A, and C61A−C91A are more significant. For C27A−C123A, there are four residues with >0.10 ppm changes in chemical shifts: Phe-5, Tyr-111, Tyr-73, and Tyr-52. Although there is only one resonance which differs by >0.10 ppm between WT and C29A−C45A, there are four residues not observable in this mutant. For C61A−C91A, there is one residue (Tyr-69) whose chemical shift difference is >0.10 ppm, whereas there are four missing residues (Try-52, Trp-3, Lys-41, and Lys-58). For these three mutants, the changes in chemical shifts are not confined to residues in the immediate environment of the mutation.

Table 1: Chemical Shifts of the Assigned Residues for WT and Mutants[a]

spin system[b]	possible assignments[c]	WT		C27A−C123A		C29A−C45A		C51A−C98A		C61A−C91A	
Fa	(F5)	6.28	7.00	6.42	6.90			6.38	7.07	6.34	7.05
		7.15		7.13				7.16		7.19	
Fb	F106	6.43	6.88			6.49	6.81	6.38	6.92	6.46	6.91
		7.26				7.29		7.30		7.29	
Fc	F94	7.10	7.35	7.11	7.35	7.14	7.39	7.11	7.35	7.13	7.36
		7.55		7.56		7.59		7.44		7.55	
Ya	Y111	6.18	6.72	6.30	6.74			6.19	6.74	6.24	6.80
Yb	Y52	6.34	6.74	6.58	6.80	6.40	6.74	6.28	6.92		
Yc	Y73	6.55	6.78	6.58	6.62	6.60	6.86	6.55	6.78	6.59	6.81
Yd	Y75	6.52	6.95	6.59	6.98	6.60	7.02	6.52	6.96	6.59	6.99
Ye	Y69	6.92	7.20	6.88	7.19	6.88	7.20	6.90	7.28	7.04	7.36
W	W3	7.30	7.34	7.29	7.33	7.28	7.30	7.32	7.36		
		7.48	7.58	7.49	7.58	7.48	7.60	7.48	7.58		
Xa	(F22)	6.75	7.04	6.80	7.11	6.83	7.08	6.75	7.07	6.78	7.06
Xb	(Y28)	6.49	7.15					6.51	7.18	6.53	7.18
ID	(I9)	0.05				0.23				0.08	
L1	(L41)	0.07		0.16				0.10			
L2	L58	0.44	0.63	0.48	0.64	0.48	0.56	0.37	0.62		
IG	I95	0.94		0.93		0.98		0.97		0.98	
A1	(A55)	1.56		1.54		1.58		1.51		1.49	
M	(M8)	1.86		1.86		1.91		1.84		1.93	

[a] The underlined are resonances that differ by >0.10 ppm between WT and mutant. [b] The designation of spin systems is based on previous work in this lab and other labs (Dupureur et al., 1992a,b; Fisher et al., 1989). [c] Parentheses indicate tentative assignments.

Table 2: Free Energies of Denaturation Induced by Gdn-HCl[a]

enzymes	$\Delta G_d^{H_2O}$ (kcal/mol)	C_m (M)	m [kcal/(mol·M)]
WT	9.5	6.8	1.41
C11A−C77A	3.3 (−6.2)	5.1	0.65
C27A−C123A	11.9 (+2.4)	6.8	1.75
C29A−C45A	9.5	6.4	1.48
C44A−C105A	6.1 (−3.4)	4.6	1.34
C51A−C98A	7.2 (−2.3)	4.4	1.64
C61A−C91A	6.8 (−2.7)	5.0	1.35

[a] Calculated from $\Delta G_d = \Delta G_d^{H_2O} - m$[Gdn-HCl], at 30 °C, pH 8.0, 10 mM borate, 0.1 mM EDTA, no Ca^{2+}. The error limit for $\Delta G_d^{H_2O}$ is estimated to be ±0.3 kcal/mol. The numbers in parentheses are differences between mutants and WT, i.e., $\Delta\Delta G_d^{H_2O}$.

In summary, the results of proton NMR analyses suggest that the conformation of C51A−C98A is little perturbed, those of C27A−C123A, C29A−C45A, and C61A−C91A are modestly perturbed, and C11A−C77A and C44A−C105A are significantly perturbed relative to the conformation of WT PLA2.

Conformational Stability. The conformational stability of mutant and WT enzymes was measured by GdnHCl-induced denaturation. The denaturation curves of mutant and WT enzymes (data not shown) display a behavior consistent with an apparent two-state folding mechanism. The denaturation data were analyzed by the equation (Pace, 1986):

$$\Delta G_d = \Delta G_d^{H_2O} - m[\text{GdnHCl}]$$

where ΔG_d is the Gibbs free energy change at various concentrations of GdnHCl, $\Delta G_d^{H_2O}$ is that at zero concentration of GdnHCl, and m is a constant related to the susceptibility of the energy toward denaturation by the denaturant. The $\Delta G_d^{H_2O}$ values, the midpoint of the denaturation curve (C_m), and the slope (m) are listed in Table 2. Deletion of the C11−C77 disulfide bond, which led to the greatest perturbation in NMR, also caused the largest decrease in the $\Delta G_d^{H_2O}$ (6.2 kcal/mol), indicating that this disulfide bond also contributes significantly to the conformational stability of PLA2. In consistence with these observations, we have found that the C11A−C77A mutant was obtained in very low yield during purification. The second-most important disulfide in structure, C44−C105, also contributes second-most importantly to conformational stability (3.4 kcal/mol). Of the remaining four disulfide mutants, C51A−C98A and C61A−C91A also display lower $\Delta G_d^{H_2O}$ (by 2.3 and 2.7 kcal/mol, respectively), while the $\Delta G_d^{H_2O}$ of the C29A−C45A mutant is as same as that of WT. Interestingly, the $\Delta G_d^{H_2O}$ of the C27A−C123A mutant *increases* by 2.4 kcal/mol.

Kinetic Analysis. The apparent kinetic constants $k_{cat,app}$ and $K_{m,app}$ of the six mutant enzymes toward DC_8PC micelles are listed in Table 3. The results from Table 3 indicate that the C29A−C45A mutant displays a lower $k_{cat,app}$ by a factor of 24 and a higher $K_{m,app}$ by a factor of 12. Since this mutant shows little changes in conformation or conformational stability, the kinetic data can be interpreted (in later sections) with confidence. Of the remaining five mutants, C44A−C105A shows a lower $k_{cat,app}$ by a factor of 8, and C27A−C123A displays a lower $k_{cat,app}$ by a factor of 4.5 and a 6-fold increase in $K_{m,app}$. The other three mutants, including C11A−C77A that displays the greatest perturbations in structural properties, show even smaller changes (less than a factor of 5 in $k_{cat,app}$ or $K_{m,app}$) relative to WT. Overall, the results suggest that, with the exception of C29−C45, the disulfide bonds are only minimally important for the catalytic function of PLA2.

Since the changes in $k_{cat,app}$ and $K_{m,app}$ toward DC_8PC micelles are generally small for the disulfide mutants, we have not further pursued detailed kinetic analyses in the scooting mode as we did in our previous studies with active site mutations. However, the possible reason for the perturbations in the kinetic behavior of C29A−C45A was further pursued with calcium binding studies as described in the following section.

Calcium Activation and Binding to C29A−C45A. The PLA2 from bovine pancreas is known to require Ca^{2+} for catalysis with a high specificity (Fleer et al., 1981; Dijkstra et al., 1981a). Since the disulfide bond C29−C45 is located near the calcium binding loop (Tyr-28 to Gly-32) (Figure 4), and since C29A−C45A is the only mutant with signifi-

Table 3: Kinetic Parameters for Hydrolysis of DC_8PC[a]

enzymes	DC_8PC micelles	
	$k_{cat,app}$ (s^{-1})	$K_{m,app}$ (mM)
WT	675	1.4
C11A−C77A	210	0.7
C27A−C123A	150	8.5
C29A−C45A	30	17
C44A−C105A	80	2.2
C51A−C98A	990	1.8
C61A−C91A	270	3.1
C29A−C45A[b]	21	2.8

[a] The error limits are estimated to be ±5% for $k_{cat,app}$ and ±15% for $K_{m,app}$. [b] At 130 mM Ca^{2+}. All other data were obtained at 25 mM Ca^{2+}.

cant changes in kinetic parameters, we further examined whether these changes could be caused by a perturbation in calcium binding. At first UV difference spectroscopy was used to determine the K_d of Ca^{2+} by monitoring the difference absorbance at 242 nm induced by Ca^{2+}, as described in Tsai et al. (1985). Both WT and C29A−C45A mutant enzymes show a typical hyperbolic profile on the plot of ΔA vs $[Ca^{2+}]$ (data not shown). However, the calcium dissociation constant of the mutant C29A−C45A deduced from the binding curve (K_d = 18 mM) is substantially higher than that of WT PLA2 (K_d = 2.0 mM). We then determined the activation constant of Ca^{2+} (K_{Ca}) for the hydrolysis of DC_8-PC micelles under a fixed substrate concentration (5 mM). The results indicate that K_{Ca} increases from 2 mM for WT (den Kelder et al., 1983) to 100 mM for the mutant.

The results of calcium binding experiments suggest that the calcium affinity of the mutant decreases by a factor of 10 or greater relative to that of WT. To determine whether the changes in the $k_{cat,app}$ and $K_{m,app}$ of the mutant are caused by the subsaturating concentration of Ca^{2+} (25 mM) used in the assays, we determined the kinetic constants at 130 mM Ca^{2+} for C29A−C45A. The results (last row in Table 3) indicate that the $K_{m,app}$ value of C29A−C45A decreases to only 2× relative to that of WT while the $k_{cat,app}$ value remains lower than WT by a factor of 32. These results taken together suggest that deletion of the C29−C45 disulfide bond possibly perturbed the calcium binding loop, which caused a decrease in the calcium binding affinity by a factor of ca. 10, and a decrease in $k_{cat,app}$ by a factor of 20−30.

DISCUSSION

The preceding sections present rigorous analyses of the structural and functional roles of each of the seven disulfide bonds of bovine pancreatic PLA2. The results suggest that at most only one disulfide bond, C84−C96, could be involved in the folding pathway. Among the other six disulfide bonds, C11−C77 is most important to the conformation and conformational stability of the enzyme, while C29A−C45A displays the greatest decrease in $k_{cat,app}$ and the greatest increase in $K_{m,app}$ toward DC_8PC micelles. Another significant finding is that deletion of the C27−C123 disulfide bond caused an unexpected *increase* in the conformational stability of the enzyme by 2.4 kcal/mol. The broader implications of our results are discussed in the following sections.

The Disulfide Bonds Are Generally Unimportant to the Folding Pathway of PLA2. It is beyond the scope of this work to examine the possible disulfide intermediates in the folding pathway, which is very complicated even for a smaller protein, bovine pancreatic trypsin inhibitor, containing only three disulfide linkages (Weissman & Kim, 1991; Creighton, 1992; Goldenberg, 1992). However, our results suggest that, of the seven disulfide bonds of PLA2, only one (C84−C96) could be essential for the folding pathway. Even in this case it cannot be completely ruled out that the inability of this mutant to refold is due to instability of the folded enzyme instead of disruption of the folding pathway.

The other six mutants all behave like WT PLA2 in the anion-exchange and cation-exchange columns during purification. In addition, the results of structural and functional analyses suggest that each of these mutants behave like WT either structurally or catalytically, or both. Thus, there is no reason to suspect major changes in their structures, such as formation of non-native disulfide bonds or existence of free cysteines. These events are likely to significantly change the structure and function of the enzymes, which is not what we have observed. The mutant enzyme with the greatest perturbation in structural properties, C11A−C77A, is fully functional catalytically. The changes in the kinetic parameters are also modest at best for the other mutants, as discussed in a later section.

These results imply that the formation of disulfide bonds in PLA2 occurs very late in the folding process, and/or that most of the disulfide bonds can be formed independently rather than sequentially. The unimportance of disulfide bonds in protein folding has its precedents: for example, ribonuclease T1 can fold properly when both of its disulfide bonds are disrupted (Pace et al., 1988).

Roles of Disulfide Bonds in the Conformational Stability and Structure. The conformational stability of PLA2 has been reported to be 7 and 9.5 kcal/mol for porcine (Pickersgill et al., 1991) and bovine enzymes (Dupureur et al., 1992a), respectively. Both values place PLA2 among the stable enzymes (Pace, 1990). Close inspection of the crystal structure of bovine pancreatic PLA2 (Dijkstra et al., 1981a,b; Noel et al., 1991) revealed that the stabilizing forces of this enzyme could involve three structural elements: an extensive hydrogen-bonding network, a few aromatic−aromatic pairs surrounding the active site, and a large number of disulfide linkages. The contributions of the first two structural elements to the structural integrity and the conformational stability of PLA2 have already been examined previously: disruption of part of the hydrogen bonding network often led to significantly perturbed NMR spectra and great decreases in the conformational stability (Dupureur et al., 1992c; Li & Tsai, 1993), while disruption of the aromatic−aromatic pairs Tyr-52/Tyr-69, Tyr-73/Tyr-75 (Dupureur et al., 1992a), Phe-22/Tyr-111, Phe-22/Phe-106, or Phe-106/Phe-5 (Dupureur et al., 1992b) led to smaller effects except for the mutants of Tyr-73. The contribution of the third structural elements, the disulfide bonds, is the subject of this work.

Disulfide bonds have been suggested to play an important role in maintaining structural integrity and protein stabilization (Pfeil, 1981). This conclusion has been supported by characterization of mutants of various proteins in which disulfide bonds have been either deleted or modified. For example, Pace et al. (1988) reported that disruption of one and two disulfide bonds in ribonuclease T1 caused decreases in conformational stability by 3.4 kcal/mol and 7.2−9.3 kcal/mol, respectively. Many other examples can be found in the reviews by Wetzel (1987) and Betz (1993). Pace (1990) suggested that disulfide bonds increase the conformational

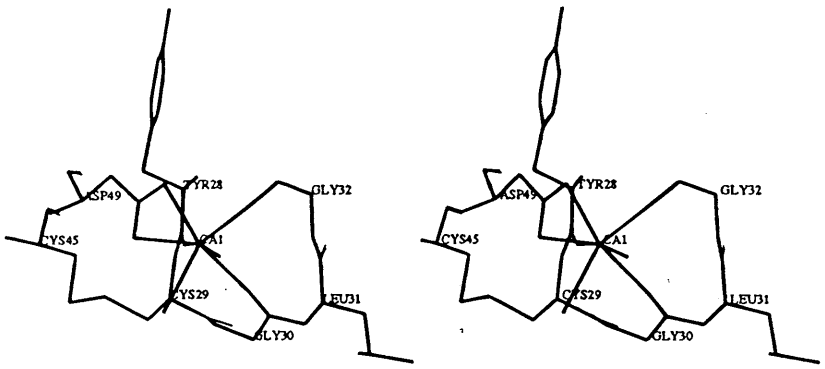

FIGURE 4: Stereoview of the C29−C45 disulfide bond and the Ca^{2+} binding site of bovine pancreatic PLA2.

stability mainly by constraining the unfolded conformations of the protein and thereby decreasing their conformational entropy.

An alternative approach to demonstrate the roles of disulfide bonds is to introduce non-native disulfide bonds into proteins by site-directed mutagenesis. This has led to enhanced stability in subtilisin BPN′ (Pantoliano et al., 1987) and T4 lysozyme (Matsumura et al., 1989a,b). However, not all of the engineered disulfide bonds enhance protein stability. In some cases, no increase or even decrease in protein stability was observed (Matsumura et al., 1989a; Wells & Powers, 1986; Wetzel, 1987; Shortle, 1989; Betz, 1993).

The thermal stability was not analyzed because the condition for *reversible* thermal denaturation could not be found for this enzyme. However, our detailed NMR analyses of the mutants allowed us to combine the conformational stability with the conformational integrity in interpreting the roles of specific disulfide linkages. As is the case in all of our previous studies, a mutant PLA2 with large perturbation in conformational stability is often highly perturbed in its NMR properties. Thus, these two structural properties usually correlate with each other qualitatively. On the basis of the results in both properties, the structural and functional roles of each of the disulfide bonds are addressed below:

C11−C77. According to the crystal structure of WT PLA2, the C11−C77 disulfide bridge is the only stabilizing force between the N-terminus and the β-wing. Therefore, removal of this disulfide bond could result in a large decrease in stability and a large conformational perturbation. In consistence with this prediction, the C11A−C77A mutant showed a large structural perturbation as evidenced by proton NMR, and a dramatically decreased conformational stability (6.2 kcal/mol). Possiblly because of the low stability, this mutant was obtained in very low yield from purification.

The C11−C77 disulfide bond is the least conserved among the seven disulfide bonds of PLA2. This seems to contradict its structural importance. However, in the cases where this disulfide bond is absent (mostly in snake venom PLA2s), it is often replaced by a salt bridge (Renetseder et al., 1985) which can also stabilize the β-wing.

C44−C105 and C51−C98. As depicted in Figure 1, C51−C98 and C44−C105 disulfide bridges connect the two main helices C and E, which consist of part of the catalytic site. Breaking C44−C105 and C51−C98 disulfide bonds resulted in a decrease of $\Delta G_d^{H_2O}$ by 3.4 and 2.3 kcal/mol, respectively. In consistence with conformational stabilities, the proton NMR property of C44A−C105A has been perturbed more extensively than that of C51A−C98A but less extensively than that of C11A−C77A, as shown in Figures 2 and 3.

In addition to these two disulfide bonds, helices C and E are also held together by a hydrogen bond between His-48 and Asp-99, which constitute the catalytic diad. It has been shown that disruption of the hydrogen bond between His-48 and Asp-99 also led to perturbations in proton NMR and in conformational stability (Li & Tsai, 1993), to even greater degrees than disruption of one of the disulfide bonds. This is possibly because the Asp-99···His-48 hydrogen bond is also part of an extensive hydrogen bonding network involving several other residues and the N-terminus (Dijkstra et al., 1981a).

C61−C91. This disulfide bond connects the short helix D with the long helix E. Perhaps because this disulfide bond is located near C51−C98, the mutants C61A−C91A and C51A−C98A behave similarly in both the conformational stability and the proton NMR property.

C29−C45. This disulfide linkage connects the calcium binding loop with one of the main helices, helix C. The mutant C29A−C45A displays unperturbed conformational stability and a slightly perturbed proton NMR property. The calcium binding ability has been hampered, as suggested by the increased calcium dissociation constant K_d and calcium activation constant K_{Ca}.

C27−C123. Surprisingly, removal of the C27−C123 disulfide bridge, which connects the C-terminus and a loop region, resulted in an increased $\Delta G_d^{H_2O}$ by 2.4 kcal/mol. It is highly unusual that a naturally occurring disulfide bond contributes negatively to the conformational stability of a protein to such an extent. Ironically, this disulfide bond is absolutely conserved among the 46 natural variants of PLA2 whose sequences are aligned by Heinrikson (1991). The catalytic function of this mutant is only very modestly perturbed as described in the following section. Thus, the possible role of the C27−C123 disulfide bond is to destabilize PLA2; it is not known whether this role has a physiological significance. Its possible catalytic significance is discussed in the next section.

Roles of Disulfide Bonds in the Activity of PLA2. Overall, the disulfide bonds are not critically important to the catalytic function of PLA2. Three mutants (C11A−C77A, C51A−C98A, and C61A−C91A), including the one displaying the greatest structural perturbations (C11A−C77A), are little perturbed in the kinetic constants (changes in $k_{cat,app}$ and $K_{m,app}$ are $<3\times$ relative to WT). In consistence with our conservative view toward site-directed mutagenesis (Tsai & Yan, 1991), we will not interpret these small changes, except to say that these disulfide bridges do not play a catalytic role.

Two other mutants, C27A−C123A and C44A−C105A, exhibit modest changes (5×−10× change in $k_{cat,app}$ or $K_{m,app}$). These marginal changes in kinetics could be meaningful, but only if the conformation is not perturbed. This is the case for C27A−C123A but not C44A−C105A. Taken together with the 2.4 kcal/mol increase in conformational stability, the results suggest that the absolutely conserved C27−C123 disulfide bond could be important for maintaining the C-terminus at a rigid conformation which is in turn important for the catalytic function of PLA2.

The effects on $K_{m,app}$ and $k_{cat,app}$ are most pronounced for the C29A−C45A mutant: $k_{cat,app}$ decreased by 24-fold and $K_{m,app}$ increased by 12-fold. These changes can be interpreted with confidence since the conformational properties of this mutant are not perturbed. Since Cys-29 is also part of the calcium binding loop (residues Tyr-28 to Gly-32), deletion of this disulfide linkage could perturb the loop and affect calcium binding. This interpretation has been confirmed by showing the substantial increases in the calcium dissociation constant K_d and the activation constant of calcium K_{Ca}. Under a saturating concentration of Ca^{2+}, the $K_{m,app}$ is no longer very different from that of WT while the $k_{cat,app}$ remains lower by a factor of 32. Thus, deletion of the C29−C45 disulfide bond is likely to perturb the binding geometry of Ca^{2+}, which caused decreases in both the calcium binding affinity and the catalytic activity. Saturation of Ca^{2+} is insufficient to restore the catalytic activity.

Conclusion. Although the roles of disulfide bonds in proteins have been investigated extensively in many systems, our system is particularly important since disulfide bonds are unusually abundant in PLA2, and the disulfide bonds in PLA2 are very highly conserved. Our study also differs from most of the other reports in that *both* structural and functional roles of each disulfide bond were investigated in detail. Our results indicate that, overall, the folding, structural, and functional roles of the individual disulfide bonds are not as important as one might predict on the basis of their abundance and sequence homology. Of the seven disulfide bonds, C84−C96 has been shown to be important to the folding process (or the folded enzyme is very unstable), C11−C77 appears to be most important to the conformational integrity and conformational stability, and C29−C45 is most important to catalysis since Cys-29 is part of the calcium binding loop. The changes in the proton NMR property and the free energy of unfolding for most mutants have been analyzed and interpreted in structural terms. The most unusual of these is the *increase* in the free energy of unfolding by 2.4 kcal/mol for the C27A−C123A mutant, which could be related to the modest changes in the kinetic constants of this mutant.

ACKNOWLEDGMENT

We thank Dr. Wayne J Becktel for his critical reading of the manuscript.

REFERENCES

Atkins, G. L., & Nimmo, I. A. (1975) *Biochem. J. 149*, 775−779.
Betz, S. F. (1993) *Protein Sci. 2*, 1551−1558.
Creighton, T. E. (1992) *Science 256*, 111−114.
Davidson, F. F., & Dennis, E. A. (1990) *J. Mol. Evol. 31*, 228−238.
de Geus, P., van den Bergh, C. J., Kuipers, O., Verheij, H. M., Hoekstra, W. P. M., & de Haas, G. H. (1987) *Nucleic Acids Res. 15*, 3743−3759.
Deng, Y., Noel, J. P., & Tsai, M.-D. (1990) *Gene 93*, 229−234.
den Kelder, G. M. D., de Haas, G. H., & Egmond, M. R. (1983) *Biochemistry 22*, 2470−2478.
Dennis, E. A. (1993) *J. Biol. Chem. 269*, 13057−13060.
Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaelen, P. J. (1978) *J. Mol. Biol. 124*, 53−60.
Dijkstra, B. W., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1981a) *J. Mol. Biol. 147*, 97−123.
Dijkstra, B. W., Drenth, J., & Kalk, K. H. (1981b) *Nature 289*, 604−606.
Dijkstra, B. W., Kalk, K. H., Drenth, J., de Haas, G. H., Egmond, M. R., & Slotboom, A. J. (1984) *Biochemistry 23*, 2759−2766.
Dupureur, C. M., Yu, B.-Z., Jain, M. K., Noel, J. P., Deng, T., Li, Y., Byeon, I.-J., & Tsai, M.-D. (1992a) *Biochemistry 31*, 6402−6413.
Dupureur, C. M., Yu, B.-Z., Mamone, J. A., Jain, M. K., & Tsai, M.-D. (1992b) *Biochemistry 31*, 10576−10583.
Dupureur, C. M., Li, Y., & Tsai, M.-D. (1992c) *J. Am. Chem. Soc. 114*, 2748−2749.
Fisher, J., Primrose, W. U., Roberts, G. C. K., Dekker, N., Boelens, R., Kaptein, R., & Slotboom, A. J. (1989) *Biochemistry 28*, 5939−5946.
Fleer, E. A. M., Verheij, H. M., & de Haas, G. H. (1981) *Eur. J. Biochem. 113*, 283−288.
Goldenberg, D. P. (1992) *Trends Biol. Sci 17*, 257−261.
Heinrikson, R. L. (1991) *Methods Enzymol. 197*, 201−214.
Li, Y., & Tsai, M.-D. (1993) *J. Am. Chem. Soc. 115*, 8523−8526.
Li, Y., Yu, B.-Z., Zhu, H., Jain, M. K., & Tsai, M.-D. (1994) *Biochemistry 33*, 14714−14722.
Liu, X., Zhu, H., Huang, B., Yu, B.-Z., Rogers, J., Kumar, A., Jain, M. K., Sundaralingam, M., & Tsai, M.-D. (1995) *Biochemistry 34*, 7322−7334.
Matsumura, M., Becktel, W. J., Levitt, M., & Matthew, B. W. (1989a) *Proc Natl. Acad. Sci. U.S.A. 86*, 6562−6566.
Matsumura, M., Signor, G., & Matthews, B. W. (1989b) *Nature 342*, 291−293.
Noel. J. P., Bingman, C., Deng, T., Dupureur, C. M., Hamilton, K. J., Jiang, R. T., Kwak, J. G., Sekharudu, C., Sundaralingam, M., & Tsai, M.-D. (1991) *Biochemistry 30*, 11801−11811.
Nozaki, Y. (1972) *Methods Enzymol. 26*, 43−50.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266−280.
Pace, C. N. (1990) *Trends Biochem. Sci. 15*, 14−17.
Pace, C. N., Grimsley, G. R., Thomson, J. A., & Barnett, B. J. (1988) *J. Biol. Chem. 203*, 11820−11825.
Pantoliano, M. W., Ladner, R. C., Bryan, P. N., Rollence, M. L., Wood, J. F., & Poulos, T. L. (1987) *Biochemistry 26*, 2077−2082.
Pfeil, W. (1981) *Mol. Cell. Biochem. 40*, 3−28.
Pickergill, R. W., Sumner, I. G., Collins, M. E., Warwicker, J., Perry, B., Bhat, K. M., & Goodenough, P. W. (1991) *FEBS Lett. 281*, 219−222.
Pieterson, W. A., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1439−1445.
Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., & Sigler, P. B. (1985) *J. Biol. Chem. 260*, 11627−11634.
Sanger, F., Niklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., & Sigler, P. B. (1990) *Science 250*, 1541−1546.
Shortle, D. (1989) *J. Biol. Chem. 264*, 5315−5318.
Taniyama, Y., Yamamoto, Y., Kuroki, R., & Kikuchi, M. (1990) *J. Biol. Chem. 265*, 7570−7575.
Thannhauser, T. W., & Scheraga, H. A. (1985) *Biochemistry 24*, 7681−7688.
Tsai, M.-D., & Yan, H. (1991) *Biochemistry 30*, 6806−6818.
Tsai, T.-C., Hart, J., Jiang, R.-T., Bruzik, K., & Tsai, M.-D. (1985) *Biochemistry 24*, 3180−3188.
Weissman, J. S., & Kim, P. S. (1991) *Science 253*, 1386−1393.
Wells, J. A., & Powers, D. B. (1986) *J. Biol. Chem. 261*, 6564−6570.
Wetzel, R. (1987) *Trends Biochem. Sci. 12*, 478−482.

BI952252W