Inactivation of a Cytosolic Phospholipase A_2 by Thiol-Modifying Reagents: Cysteine Residues as Potential Targets of Phospholipase A_2†

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ABSTRACT: The cytosolic phospholipase A_2 ($cPLA_2$) from the human monocytic cell line U937 contains nine cysteine residues and is subject to oxidation. Iodoacetamide and 5,5′-dithiobis(2-nitrobenzoic acid) were used to explore the susceptibility of cysteine residues to thiol modification agents as outlined in Schemes 2 and 3. In the absence of thiol reducing agents such as DTT, $cPLA_2$ takes up only 2.8 equiv of [1-^{14}C]iodoacetamide at pH 8.03/37 °C. With DTT present, $cPLA_2$ is in its fully reduced form, and 4–5 equiv of acetamide are taken up without altering enzyme activity to give IA–$cPLA_2$. A single equivalent of DTNB suffices to inactivate IA–$cPLA_2$, giving a TNB-labeled enzyme, with the loss of activity correlating with release of an equivalent of 5-thio-2-nitrobenzoate. The TNB-labeled enzyme is quite stable up to 33 °C; enzyme activity is recoverable with DTT, even after this disulfide–enzyme adduct is incubated with iodoacetamide at pH 9.5, conditions that inactivate the free enzyme. At pH 9.5/37 °C, a single equivalent of ^{14}C-labeled iodoacetamide is incorporated by IA–$cPLA_2$ concomitant with complete loss of enzyme activity. Amino acid analysis of the ^{14}C-labeled enzyme indicates that only cysteine residues are labeled. Lys-C digestion of labeled enzyme with 2 M guanidine at pH 8.0 yields a 40-mer peptide. Amino acid sequencing establishes that the label resides primarily in Cys^{324}, although Cys^{331} is also labeled. These results identify a region of the enzyme that is susceptible to labeling by group modification reagents and may represent a suitable target for small molecule inhibitors.

Phospholipase A_2 (PLA_2)[1] catalyzes the hydrolysis of the ester function at the *sn*-2 position of its phospholipid substrates, resulting in fatty acid and lysophospholipid products (Dennis, 1983; Waite, 1987). The well-known low molecular mass (14 kDa) secretory PLA_2 enzymes have been extensively studied over the past decade, and relevant three-dimensional structures have been determined by X-ray crystallography [for reviews see Dennis (1987), Jain and Berg (1989), Scott et al. (1990), and Ramirez and Jain (1991)].

Recently, a high molecular weight cytosolic PLA_2 ($cPLA_2$) has been purified from the human monocytic cell line U937 (Clark et al., 1990; Diez & Mong, 1990; Kramer et al., 1991) and cloned and expressed (Clark et al., 1991; Sharp et al., 1991). Several other high molecular weight PLA_2 enzymes have been purified from the macrophage cell line RAW 264.7 (Leslie et al., 1988), rat kidney (Gronich et al., 1990) and mouse spleen (Wijkander & Sundler, 1991). Although both high and low molecular weight forms of PLA_2 enzymes hydrolyze ester linkages of phospholipids at the *sn*-2 position preliminary reports of the cytosolic enzyme reveal that it differs in a number of respects from the secretory forms. Besides dramatic differences in size ($cPLA_2$ has $M_r \approx 85\,000$ as compared to $M_r \approx 14\,000$ for secretory enzymes), there is no apparent amino acid sequence homology between the two enzyme types. In addition, there appears to be a separate calcium-binding domain within $cPLA_2$ which is homologous to that of PKC and PLC (Clark et al., 1991). Also, as opposed to secretory PLA_2, which utilizes Ca^{2+} for catalysis, $cPLA_2$ enzymes use Ca^{2+} to facilitate binding to aggregated substrate (Yoshihara & Watanabe, 1990; Zupan et al., 1991; Wijkander & Sundler, 1992). Whereas the seven cystine disulfide bonds of secretory PLA_2 enzymes are known to contribute to the great stability of these proteins, under physiological conditions, the nine cysteine residues of $cPLA_2$ must all be in the thiol form owing to the reducing environment of the cytosol.

An important difference between the two enzyme types is their specificity for the *sn*-2 fatty acid component of phospholipid substrates. Intense interest in cytosolic PLA_2 derives from its specificity in hydrolyzing arachidonyl-containing phospholipids to furnish arachidonic acid, which is further elaborated to a number of potent inflammatory mediators (Irvine, 1982; Larsen & Henson, 1983; Samuelsson et al., 1987). By contrast, secretory enzymes are less sensitive to the fatty acid chains at the *sn*-2 position of their phospholipid substrates. The distinct substrate specificities afford an opportunity to preferentially inhibit $cPLA_2$, thereby selectively blocking the cascade of inflammatory mediators generated specifically by this enzyme and enabling an assessment of its role in inflammatory processes.

In this study, with group modification reagents, we sought to obtain fundamental information about the chemical properties of specific amino acid residues that give a clear and

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[1] Abbreviations: BSA, bovine serum albumin; BMH, bis(maleimido)-hexane; CMC, carboxymethylated cysteine; $cPLA_2$, cytosolic phospholipase A_2; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; IA, iodoacetamide; IAA, iodoacetic acid; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; MS, mass spectroscopy; MMTS, methyl methanethiolsulfonate; NBM, *N*-benzylmaleimide; NEM, *N*-ethylmaleimide; PAPC, 1-palmitoyl-2-archidonylphosphatidylcholine; [^{14}C]PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonylphosphatidylcholine; pCMB, *p*-(chloromercuri)benzoic acid; PLA_2, phospholipase A_2; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TNB, 5-thio-2-nitrobenzoic acid; TX-100, Triton X-100.

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unambiguous picture of their potential for labeling. We report herein that specific cysteine residues of $cPLA_2$ can be modified by either DTNB or iodoacetamide, resulting in complete loss of enzyme activity, thereby pinpointing a specific region of the enzyme that may be worth targeting using small molecule inhibitors.

MATERIALS AND METHODS

Materials. [1-^{14}C]Iodoacetamide (58 mCi/mmol) was purchased from Amersham. [^{14}C]PAPC (53 mCi/mmol) was obtained from New England Nuclear. PAPC was acquired from Avanti Polar Lipids. DTNB, DTT, and iodoacetamide were purchased from Sigma Chemical Co. Endoproteinase Lys-C was purchased from Boehringer Mannheim. Sephadex G-50 was bought from Pharmacia Fine Chemicals. All other chemicals were of the highest commercial grade. The C_{18} reversed-phase HPLC column was purchased from Vydac.

Human cytosolic PLA_2 from U937 was purified from expressed COS cells containing the cloned $cPLA_2$, gene, pMT2-$cPLA_2$, according to the published procedure (Clark et al., 1991). The purified enzyme was stored at −80 °C in 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $CaCl_2$, and 5 mM DTT, which was 50% w/v glycerol, and was more than 90% pure as judged from SDS–PAGE. This form of the enzyme is referred to as $cPLA_2$, whereas IA–$cPLA_2$ is a carboxamidomethylated form of $cPLA_2$ (vide infra) which is used extensively in our studies. Enzyme concentrations were determined spectrophotometrically at 280 nm using the extinction coefficient $\epsilon^{1\%} = 0.745$ (James Clark, Genetics Institute). The protein concentrations were also determined according to the method of Bradford using BSA as a standard and the Bio-Rad protein assay kit. The standard buffers used in our studies are composed of 100 mM NaCl, 10 mM $CaCl_2$, and 30% w/v glycerol with the following buffering reagents: pH 6–6.9, 50 mM MES; pH 7–7.9, 50 mM HEPES; pH 8–8.9, 50 mM Tris; and pH 9–10.6, 100 mM glycine.

Preparation of Alkyl Analogs of DTNB. 5-(Alkyldithio)-2-nitrobenzoic acids were prepared by reacting the appropriate alkanethiols with DTNB in the presence of triethylamine (NEt_3). For example, 0.1 mL of hexanethiol (0.71 mmol) was added to 300 mg (0.76 mmol) of DTNB in 30 mL of CH_2Cl_2 containing 0.5 mL of NEt_3 at room temperature with stirring. The reaction mixture turned yellow and was left overnight, after which time 20 mL of 5% HCl was added. The organic phase was separated and washed with 20 mL of water and 20 mL of brine, dried over anhydrous $MgSO_4$, and then concentrated to an oil. Purification by flash silica gel chromatography eluting with ethyl acetate yielded 100 mg of 5-(hexyldithio)-2-nitrobenzoic acid: ^{1}H NMR (80 MHz, $CDCl_3$) δ 0.9 (br t, 3H, CH_3), 1.0–1.9 (br m, 8H, CH_2), 2.8 (t, 2H, CH_2S), 7.7–8.0 (m, 3H, Ph), 10.2 (br, 1H, CO_2H); MS (EI) m/z 315 (M^+), 231, 213, 85.

Enzyme Assays. Enzymatic activity was assayed in a mixed micelle system using 1-palmitoyl-2-[1-^{14}C]-arachidonylphosphatidylcholine as substrate as described previously (Clark et al., 1990), with modifications. The assay medium was prepared by drying 70 μL of PAPC (1 mg/mL) in 1:1 ethanol/toluene and 30 μL of [^{14}C]PAPC (0.74 mg/mL; 53 mCi/mmol) in chloroform with a stream of nitrogen and then diluting this mixture to 6 mL with a solution of 80 mM glycine (pH 9.0), 1 mM $CaCl_2$, and 2.67 mM deoxycholate in 70% w/v glycerol, to give a final concentration of phospholipid of 20 μM (13 mCi/mmol). The substrate stock solution was vortexed and then sonicated on ice for 1 min using a Braun-Sonic 1510 tip sonicator at 400 W to form mixed micelles. In a typical assay, 30 ng of enzyme was added to 100 μL of substrate solution (with or without inhibitor present) at 37 °C and then vigorously vortexed to achieve homogeneity. After 10 min, the reaction was quenched with 200 μL of the stop solution (70:30:1 mixture of hexane/1,4-dioxane/acetic acid). The entire mixture was then loaded onto a column containing 1.5 mL of silica gel 60, which had been prewashed with 1 mL of 20 μg/mL arachidonic acid in hexane. The column was eluted three times with 2 mL of the stop solution on a Speed Mate-30 vacuum manifold. The eluate was collected directly in a scintillation vial, mixed with 8 mL of ScintiVerse I (Fisher Scientific), and then counted in a Beckman LS-7500 scintillation counter. Under these assay conditions, the specific activity of $cPLA_2$ was 1 u/mg (1 u = release of 1 μmol of arachadonic acid/min).

Preparation of Iodoacetamide-Labeled $cPLA_2$ at pH 8.0 (IA–$cPLA_2$). For inactivation kinetics and stoichiometric determinations, the enzyme was pretreated with iodoacetamide at pH 8.0 to block chemically reactive cysteines. Such treatment did not alter the activity of the enzyme. In these experiments, the enzyme ($cPLA_2$, 2 mg/mL) was incubated with 4 mM DTT in the standard buffer (pH 8.0) for 20 min at room temperature, and then iodoacetamide was added to the medium to a concentration of 23 mM and the mixture was incubated for 3 h at 37 °C. To remove excess reagents, the mixture was passed through a Sephadex G-50 column (2 mL) that was pre-equilibrated with 6 mL of the standard (pH 7.5) buffer. The eluate containing the enzyme was stored at −20 °C. Enzyme treated as above will be referred to as IA–$cPLA_2$ in text and possesses the same specific activity as $cPLA_2$ in our mixed micelle assay.

Inactivation of $cPLA_2$ or IA–$cPLA_2$. The inactivation of either $cPLA_2$ or IA–$cPLA_2$ by DTNB was carried out in the standard buffer (pH 7.5) at 25 °C. Unless otherwise specified, the reaction mixture also contained 250 μM TX-100. Immediately before treatment with DTNB, DTT was removed from the stock solution of enzyme by gel filtration (Sephadex G-50). Aqueous DTNB stock solution was then added to enzyme, and aliquots of the reaction mixture were removed at various time intervals and assayed for activity with the mixed micelles. The control sample contained enzyme under the same conditions, without inactivator. To obtain first-order rate constants of inactivation, the residual enzyme activity in the reaction mixture measured at various times was fitted to the following simple exponential decay equation using the nonlinear regression program (Enzfit):

$$y = y_0 \exp(-k_{obs}t) \quad (1)$$

The value y_0 is the enzyme activity at time zero, y is residual activity time t, and k_{obs} is the observed first-order rate constant of inactivation. Unless otherwise specified, the second-order rate constants were calculated by dividing the observed first-order rate constants by the corresponding inhibitor concentrations.

The inactivation of IA–$cPLA_2$ by iodoacetamide was carried out at 37 °C under the same conditions as described above for DTNB except that 100 mM glycine (pH 9.5) instead of HEPES was used in the reaction buffer. Kinetic data were also analyzed using eq 1 as described above.

Determination of the Stoichiometry for the Inactivation of IA–$cPLA_2$ by DTNB. The stoichiometry of inactivation IA–$cPLA_2$ by DTNB was quantitated by correlating the amount of 5-thio-2-nitrobenzoate released (λ_{max} = 412 nm, ϵ = 13 600 M^{-1} cm^{-1}) with the loss of enzyme activity. To 380 μL of mg/mL IA–$cPLA_2$ in standard buffer (pH 7.5), with 250 μM

TX-100 at 25 °C, was added 20 μL of 10 mM DTNB in 50 mM HEPES (pH 7.5) to give a final concentration of 500 μM DTNB. The absorbance at λ = 412 nm was monitored at various time intervals, and aliquots of the reaction mixture were removed to assay enzyme activity. The negative control, lacking enzyme, showed no significant change in absorbance at λ = 412 nm over the reaction course.

DTNB inactivation of IA–cPLA$_2$ was carried out under the same conditions for isolation and characterization of the modified enzyme. The reaction was followed until >95% enzyme activity was inhibited, and then the mixture was passed through a Sephadex G-50 column which was pre-equilibrated with the standard buffer (pH 7.5). The DTNB-inactivated IA–cPLA$_2$ was stored at −20 °C.

Determination of the Stoichiometry of Iodoacetamide Labeling. The stoichiometry of iodoacetamide labeling was determined using [1-^{14}C]iodoacetamide. Because of the uptake of iodoacetamide by cPLA$_2$ prior to inactivation, the stoichiometry of cPLA$_2$ was determined in two stages: at pH 8.0/37 °C to produce active IA–cPLA$_2$ and at pH 9.5/37 °C to produce radiolabeled, inactive enzyme.

(a) Stage I. The number of cysteine residues susceptible to labeling without loss of enzyme activity was determined using cPLA$_2$ (either in the presence or in the absence of DTT) by measuring ^{14}C incorporation in enzyme at pH 8.0 (conditions that do not lead to inactivation).

cPLA$_2$ (56 μL of 0.92 mg/mL) in the standard buffer (pH 8.0) with 1.4 mM DTT was mixed with 24 μL of 40 mM [^{14}C]iodoacetamide (7.25 mCi/mmol) in the above buffer without DTT and then incubated at 37 °C. Aliquots of 8 μL of the reaction mixture were removed at time intervals and added to 50 μL of 1 mg/mL BSA. The enzyme–BSA mixture was then precipitated with 100 μL of ice-cold 30% TCA and loaded onto a Whatman fiberglass filter paper in a suction filter funnel. The filter paper was washed eight times with 4 mL of ice-cold 8% TCA, once with 2 mL of ice-cold absolute ethanol, and then counted for radioactivity. The enzyme activity was monitored at time intervals by assaying a separate sample under the identical conditions in a parallel experiment, except that nonisotopically labeled iodoacetamide was used.

The [^{14}C]iodoacetamide labeling of enzyme in DTT-free solvent was carried out as follows. DTT-free cPLA$_2$ (15 μL of 1.7 mg/mL) in the standard buffer (pH 8.0) was mixed with 15 μL of 20 mM [^{14}C]iodoacetamide (7.25 mCi/mmol) in the same buffer and incubated at 37 °C. Aliquots of the reaction mixture (3.5 μL) were removed from the mixture, precipitated, and counted as described above.

(b) Stage II. The stoichiometry of iodoacetamide inactivation of the IA–cPLA$_2$ enzyme was determined at pH 9.5 and 37 °C. IA–cPLA$_2$ (42 μL of 0.37 mg/mL) in standard buffer with 0.25 mM TX-100 was mixed with 8 μL of 10 mM [^{14}C]-iodoacetamide (58 mCi/mmol) and then incubated at 37 °C, precipitated with TCA, and counted for ^{14}C incorporation.

Determination of Thiols in IA–cPLA$_2$ under Denaturing Conditions. The number of thiols in IA–cPLA$_2$ under denaturing conditions was determined at pH 7.5 and 37 °C. IA–cPLA$_2$ (400 μL of 1.17 mg/mL) in standard buffer with 4 M guanidine hydrochloride was mixed with 6 μL of 5 mM $C_{10}H_{21}S$–TNB. Absorbance at λ = 412 nm was followed until no further increase was observed (10 min). The number of thiols modified was calculated from the released 5-thio-2-nitrobenzoate molecules with ε = 13 600 M^{-1} cm^{-1} at λ = 412 nm. A blank control was run without the enzyme under the same conditions.

Identification of ^{14}C-Labeled Carboxamidomethylated Cysteine. The ^{14}C-labeled carboxamidomethylated enzyme was prepared by adding [^{14}C]iodoacetamide to IA–cPLA$_2$ under the conditions of enzyme inactivation at pH 9.5. Reaction mixture containing 0.5 mg of the resulting fully inactivated cPLA$_2$ in 250 μL was precipitated with 500 μL of ice-cold 30% TCA and washed three times with 300 μL of ice-cold 8% TCA and once with 300 μL of ice-cold water. To fully label residual cysteines, the precipitated enzyme was redissolved in 50 μL of 50 mM Tris (pH 8.0), 4 M guanidine, and 15 mM iodoacetamide and incubated at 37 °C for 1 h. The enzyme solution was then diluted with 150 μL of 50 mM Tris (pH 8.0) and precipitated with 400 μL of 30% TCA. After three washes with 300 μL of ice-cold 8% TCA and one wash with 300 μL of ice-cold water, the labeled enzyme was resuspended in 50 μL of buffer containing 50 mM Tris (pH 8.0), 6 M guanidine, and 10 mM EDTA. This solution was incubated at 50 °C for 30 min to completely dissolve and denature the enzyme and then diluted with 100 μL of 50 mM Tris (pH 8.0) and 10 mM EDTA to give a final concentration of 2 M guanidine. Endoproteinase Lys-C (10 μg) was then added, and the digestion was performed at 37 °C for 24 h. Peptide separation was accomplished with a Vydac C_{18} hydrophobic reversed-phase column (4.6 × 250 mm). A 70-min linear gradient was used, starting from 90% solvent A (0.1% TFA in H_2O) and 10% solvent B (0.1% TFA in acetonitrile) to 50% A and 50% B at a flow rate of 1 mL/min. Absorbance of the eluted peptides was monitored at λ = 215 nm. Peptide fractions were manually collected, and $^1/_{30}$ of each fraction was counted for radioactivity. The fractions richest in radioactivity were combined, lyophilized, resuspended, and refractionated on the same column, using a 40-min linear gradient from 70% A and 30% B to 60% A and 40% B, at a flow rate of 0.5 mL/min. The purified peptide was lyophilized and sequenced with an Applied Biosystems Instruments Type 478 protein sequencer. A small fraction was removed from each cycle to determine the ^{14}C content.

For amino acid analysis of ^{14}C-labeled carboxamidomethylated enzymes, samples were prepared under the conditions of stoichiometric determination with the use of 0.13 mg of enzyme at pH 8.0 and 0.2 mg of enzyme at pH 9.5, as described in the above section. The labeled proteins were precipitated, washed, and treated with iodoacetamide as previously described. The precipitated proteins were then dissolved in 30% acetonitrile and submitted for amino acid analysis by Pharmacia Biotechnology Service Centre at Toronto. A fraction of HPLC eluate was collected at each 0.5-min interval and counted for ^{14}C radioactivity.

RESULTS

Reaction of cPLA$_2$ with Iodoacetamide at pH 8: Labeling of Reactive Thiol Residues To Give IA–cPLA$_2$. The cDNA sequence indicates that there are a total of nine cysteines in cPLA$_2$. In the reducing environment of the cytosol, it is very likely that all of the cysteines of cPLA$_2$ are in thiol form. The isolated enzyme is more stable in the presence of reducing agents such as DTT or β-mercaptoethanol. Upon removal of disulfide reducing agents, up to 40% of the enzymatic activity is lost, which can be recovered by adding 1 mM DTT to the enzyme solution.

Treatment of cPLA$_2$ with up to 10 mM iodoacetamide for as long as 5 h at pH 8.0, 37 °C, does not affect enzyme activity. Typically, specific activity of 1.0 ± 0.2 μmol min^{-1} mg^{-1} can be observed after this treatment. However, alkylation of the enzyme does occur as measured by incorporation of radiolabel

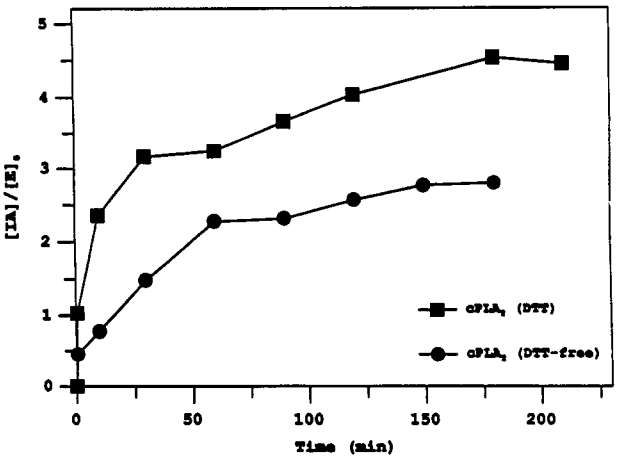


FIGURE 1: Time course of the incorporation of iodoacetamide by cPLA$_2$ at pH 8.0. Iodoacetamide (10 mM) was incubated with 0.85 mg/mL of DTT-free cPLA$_2$ (solid circles) at 37 °C, pH 8.0, and the stoichiometry of the iodoacetamide reaction with the enzyme at various time intervals was measured as described under Materials and Methods. Alternatively, 12 mM solid iodoacetamide was incubated with 0.64 mg/mL of enzyme which contained 1.4 mM of DTT (solid squares). The point at the origin is the control without iodoacetamide.

by the enzyme upon treatment with [^{14}C]iodoacetamide. The number of residues labeled depends upon the state of oxidation of the enzyme. Figure 1 shows the time course of the reaction of enzyme at pH 8.0 and 37 °C with [^{14}C]iodoacetamide in the presence or absence of DTT. In the absence of DTT, approximately 2.8 equiv of acetamide is incorporated per equivalent of enzyme (solid circles). However, if the same enzyme solution is pretreated with 1.4 mM DTT at room temperature, followed by incubation with 12 mM iodoacetamide at 37 °C, 4.5 equiv of acetamide is incorporated (solid squares). The rate of IA incorporation in the presence of DTT shows an initial burst which is equivalent to the uptake of 1.7 equiv of iodoacetamide. The reaction then slows to a rate similar to that observed for the DTT-free enzyme.

On the basis of the difference in the total number of equivalents of reagent incorporated during the two labeling experiments, it appears that the two most reactive cysteines rapidly form a disulfide bond in the absence of thiol reducing agent. Treatment of cPLA$_2$ with iodoacetamide (in the presence of DTT) at pH 8.0 results in an acetamidomethylated form of the enzyme, IA–cPLA$_2$, which has essentially the same activity as cPLA$_2$, but is more stable, and is the form of the enzyme we chose for subsequent studies.

Inactivation of cPLA$_2$ and IA–cPLA$_2$ by DTNB. Whereas iodoacetamide does not affect enzyme activity at pH 8.0, despite carboxamidomethylation of a number of cysteine residues, incubation of the cytosolic enzyme with DTNB in the pH range 6.0–9.5 results in time-dependent loss of enzyme activity. The observed inactivation rates are equal for cPLA$_2$ and IA–cPLA$_2$. Figure 2A shows the time course of enzyme inactivation at various concentrations of DTNB. The data are fit to eq 1 by nonlinear regression analysis to obtain the apparent first-order rate constant of inactivation k_{obs} as represented by the curves. The control, enzyme in buffer lacking DTNB, shows no significant loss of activity during a parallel experiment. The inactivation rate is clearly dependent upon the concentration of inhibitor and does not reach saturation at concentrations up to 5 mM DTNB. Figure 2B is a plot of the first-order rate constants of inactivation versus DTNB concentration. The rate constant shows an initial hyperbolic increase followed by a linear increase. The data

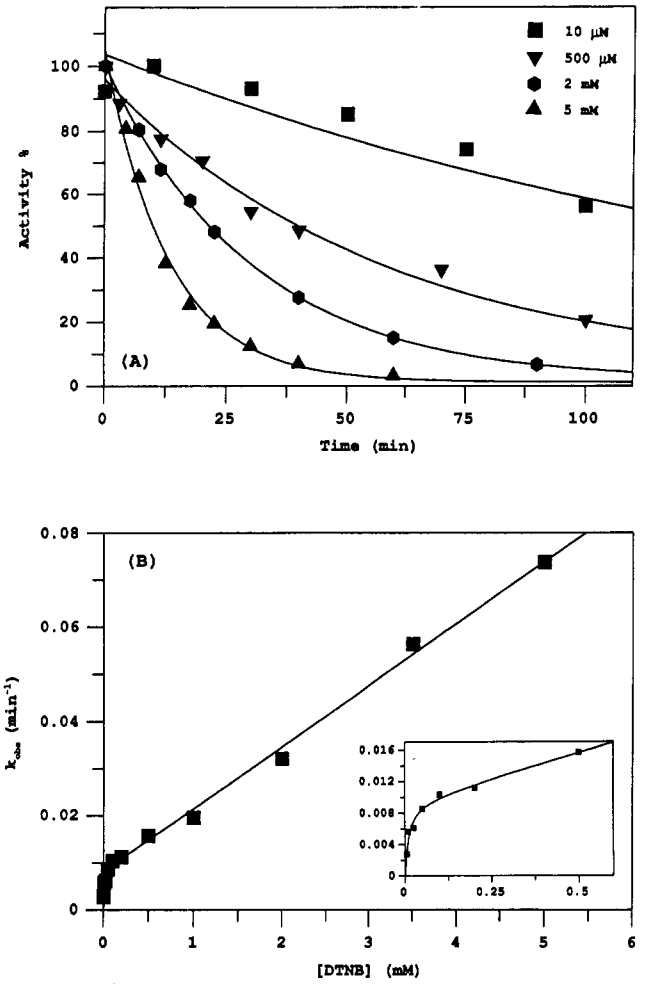


FIGURE 2: Concentration dependence of the inhibition of IA–cPLA$_2$ by DTNB. IA–cPLA$_2$ (0.03 mg/mL) was incubated with DTNB at 25 °C, pH 7.0. Shown in (A) are the time courses of the inhibition at various DTNB concentrations. The data were fit to eq 1 by nonlinear regression analysis, and the lines represent the best fit parameters for the corresponding data. (B) is the plot of k_{obs} versus the corresponding DTNB concentration. The data were fit to eq 2, and the line represents the best fit parameters of $a = 0.0084 \pm 0.0009$ min^{-1}, $b = (7.2 \pm 4) \times 10^{-6}$ M, and $c = 13.1 \pm 0.3$ M^{-1} min^{-1}. The inset in (B) is the same plot in the range between 0 and 0.5 mM iodoacetamide.

can be best fit to

$$k_{obs} = a[I]/(b + [I]) + c[I] \quad (2)$$

with the best fit parameters of $a = 0.0084 \pm 0.0009$ min^{-1}, $b = (7.2 \pm 4) \times 10^{-6}$ M, and $c = 13.1 \pm 0.3$ M^{-1} min^{-1}. To fit the data, we have assumed Scheme 1, in which a minor form of the enzyme (E′) reacts with DTNB much faster than the major form (E). EI is a noncovalent complex between the major form of the enzyme and inhibitor, and EI* is the covalent inactivated form.

Assuming the steady-state approximation for EI and E′, then the corresponding rate equations are

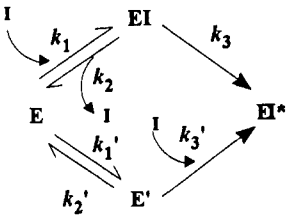
$$-d[EI]/dt = 0 = (k_2 + k_3)[EI] - k_1[I][E] \quad (3)$$

$$-d[E']/dt = 0 = (k_2' + k_3'[I])[E'] - k_1'[E] \quad (4)$$

and

$$-d[E]/dt = d[EI^*]/dt = k_3[EI] + k_3'[I][E'] \quad (5)$$

Scheme 1



Rearranging eqs 3 and 4 and then substituting into eq 5 gives

$$-\mathrm{d}\ln[\mathrm{E}]/\mathrm{d}t = \frac{k_1k_3}{k_2+k_3}[\mathrm{I}] + \frac{k_1'[\mathrm{I}]}{(k_2'/k_3') + [\mathrm{I}]} \quad (6)$$

Integrating eq 6 gives [E] as

$$[\mathrm{E}] = [\mathrm{E}_0]\exp -[(k_1k_3/k_2 + k_3)[\mathrm{I}] + k_1'[\mathrm{I}]/((k_2'/k_3') + [\mathrm{I}])]t \quad (7)$$

where $[E_0]$ is the free enzyme concentration at $t = 0$. Comparing eq 7 with eq 1

$$k_{obs} = \frac{k_1k_3}{k_2+k_3}[\mathrm{I}] + \frac{k_1'[\mathrm{I}]}{(k_2'/k_3') + [\mathrm{I}]} \quad (8)$$

Figure 3 shows the pH dependence of enzyme inactivation of IA–cPLA$_2$ from pH 6.0 to 9.5 at 25 °C with 0.5 mM DTNB (the enzyme is not stable at pH lower than 6). The stoichiometry of enzyme inactivation by DTNB was determined by following the absorbance at λ = 412 nm, corresponding to the release of TNB. The number of equivalents of TNB released should be directly related to the number of enzyme cysteines modified by DTNB. As indicated in Figure 4, the complete loss of enzymatic activity of IA–cPLA$_2$ upon treatment with DTNB at pH 7.5 corresponds to the release of 1.14 TNB equiv per enzyme, indicating that modification of a single cysteine residue per equivalent of enzyme is responsible for the inactivation.

Recovery of Enzyme Activity from TNB–cPLA$_2$ Using DTT. If the inactivation of IA–cPLA$_2$ is due to thiol–disulfide exchange reaction between a cysteine residue of the enzyme and DTNB, enzyme activity should be restored to its original level upon treatment with disulfide reducing agents such as DTT or β-mercaptoethanol. Indeed, as shown in Table 1, the activity of DTNB-inactivated enzyme could be completely recovered when incubated with concentrations of DTT at pH 8.0 and room temperature for 20 min. The reversibility of the inactivation conforms to a pattern anticipated for modification of a cysteine residue by DTNB through a thiol–disulfide exchange reaction.

Inactivation of cPLA$_2$ by Analogs of DTNB and Other Thiol Modification Reagents. Since the modification of a specific cysteine residue of cPLA$_2$ can result in complete loss of the enzyme activity, the effects of other known thiol modification reagents on cPLA$_2$ activity were examined. It was found that standard thiol modification reagents inhibit enzyme activity in a time-dependent manner. Table 2 summarizes the first-order rate constants for the inactivation of enzyme by these reagents, together with the inhibitor concentrations used.

cPLA$_2$ prefers lipid substrates with long-chain fatty acids at the *sn*-2 position (Diez et al., 1992). A series of DTNB analogs, in which one of the polar aromatic rings was replaced by alkyl chains of various lengths, were synthesized and tested as cPLA$_2$ inhibitors. The results are depicted in Figure 5. In this series, the rate of enzyme inactivation increases with

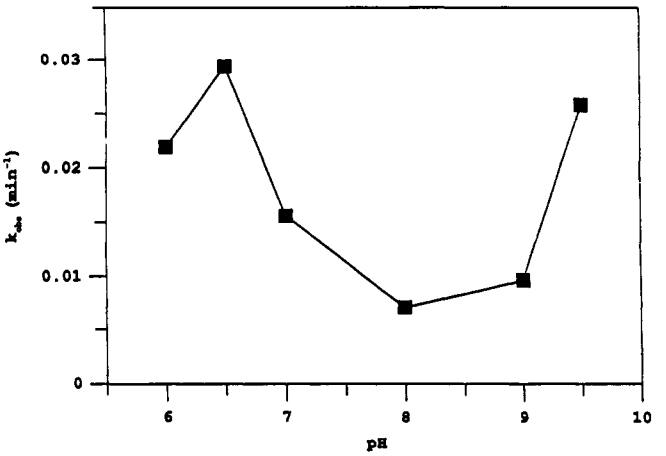


FIGURE 3: pH dependence of DTNB inactivation of IA–cPLA$_2$. IA–cPLA$_2$ (0.15 mg/mL) was incubated with 0.5 mM DTNB at various pH values, 25 °C. The k_{obs} was calculated from eq 1.

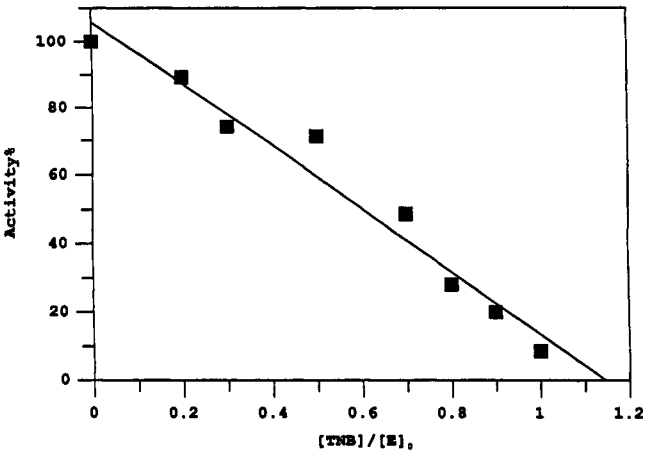


FIGURE 4: Titration of IA–cPLA$_2$ by DTNB. The inhibition reaction was carried out at 25 °C, pH 7.5, with 1 mg/mL IA–cPLA$_2$ and 0.5 mM DTNB. At time intervals, aliquots were removed to assay the remaining enzyme activity, while the corresponding TNB released was determined by absorbance at 412 nm, as described under Materials and Methods.

Table 1: Concentration Dependence of Restoration of TNB–cPLA$_2$ Activity[a]

[DTT] (mM)	activity (% control)	[DTT] (mM)	activity (% control)
0	2.4	0.8	93
0.2	67	2.0	94

[a] TNB-labeled IA–cPLA$_2$ (0.05 mg/mL) was incubated with various concentrations of DTT at pH 8.0, 25 °C, for 20 min and then immediately assayed for enzyme activity. The activity was then compared with that of native enzyme.

increasing chain length, indicating that the lipophilic component of the inhibitor can facilitate its binding to the enzyme.

Inactivation of the Enzyme by Iodoacetamide. On the basis of the DTNB inactivation results, it is apparent that modification of a single cysteine can result in the loss of the cPLA$_2$ activity. However, as noted previously, iodoacetamide, a common cysteine modification reagent, did not inhibit the enzyme activity at pH 8.0, even at 10 mM concentration. While there was no observable inhibition at pH lower than 8.5, an appreciable amount of enzyme inhibition is observed at pH 9.0 and above.

Figure 6A shows the time course of the inhibition of IA–cPLA$_2$ and the incorporation of labeled reagent by enzyme at pH 9.5 in the presence of 1.6 mM iodoacetamide at 37 °C.

Table 2: Inhibitory Effects of Some Typical Thiol Modification Reagents[a]

inhibitor	[I] (mM)	condition (pH/°C)	k_{obs} (min^{-1})
IA	10	9.5/37	0.15
IAA	10	9.5/37	0.071
IAEDANS	1	9.5/37	0.15
DTNB	5	7.0/25	0.074
MMTS	6	7.0/25	0.012
NEM	15	7.5/37	0.023
NBM	5	7.5/37	0.044
BMH	0.2	7.5/37	0.14
$HgCl_2$	0.7	7.5/37	0.46
pCMB	0.4	7.5/37	0.23

[a] All of the inhibition experiments were carried out in the presence of 250 μM TX-100 with 0.05 mg/mL of IA–cPLA$_2$. The time course of the inhibition was fit to eq 1 to obtain the first-order rate constants (k_{obs}).

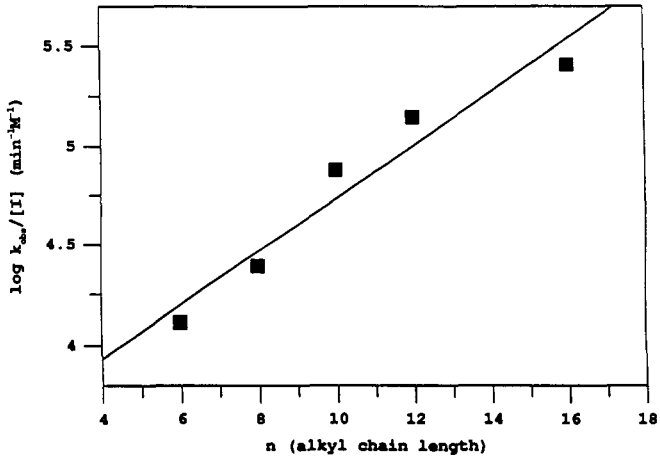


FIGURE 5: Rates of inhibition of cPLA$_2$ by alkyl–S–TNB analogs versus alkyl chain length. The inhibition was carried out at pH 6.0, 37 °C, and k_{obs} was obtained by fitting the data to eq 1. The second-order rate constants, k_{obs}/[I], were calculated from k_{obs} and inhibitor concentration in the range 5–25 μM. The line is the linear fitting of the data with a correlation coefficient of 0.94.

The curve represents the best fit of the inhibition data (solid squares) to eq 1 with a pseudo-first-order rate constant k_{obs} of 0.05 min^{-1}. It is clear from Figure 6A that the inactivation of the enzyme is closely correlated with the covalent attachment of the reagent as judged by the correspondence between incorporation of radiolabel (open squares) and loss of enzyme activity. These results strongly suggest that a single enzyme residue has been modified by iodoacetamide concomitant with the loss of enzyme activity.

Figure 6B shows how the concentration of iodoacetamide affects the apparent first-order rate constant for inactivation of IA–cPLA$_2$ at pH 9.5/37 °C. Assuming a rapid binding of enzyme and inhibitor followed by a slow chemical reaction, the data were fit to

$$k_{obs} = k_{inact}[I]/(K_i + [I]) \quad (9)$$

and the curve represents the best fit parameters of k_{inact} = 0.2 min^{-1} and $K_i = 2.5 \times 10^{-3}$ M. The second-order inactivation rate constant k_{inact}/K_i = 80 M^{-1} min^{-1} is relatively low for iodoacetamide modification of a protein cysteine (Lundblad & Noyes, 1984) but is not without precedent (Todd & Hansinger, 1991).

The pH profile of the inactivation of PLA$_2$ by iodoacetamide is shown in Figure 7 and is similar to the dependence of enzyme activity on pH in the mixed micelle assay, in that both show increased activity at pH >9.5. Unfortunately, a reliable determination of the pK_a of iodoacetamide inactivation could not be accurately made owing to the instability of the enzyme at pH >10.5.

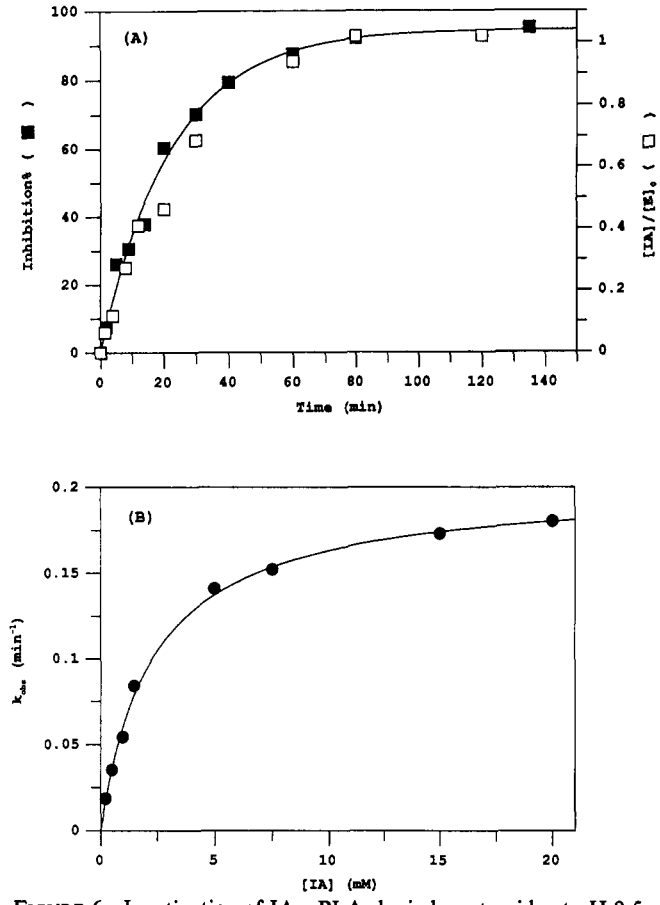


FIGURE 6: Inactivation of IA–cPLA$_2$ by iodoacetamide at pH 9.5. (A) Time course of the inactivation of IA–cPLA$_2$ (0.31 mg/mL) incubated with 1.6 mM iodoacetamide at 37 °C, pH 9.5. The percentage of inactivation was calculated from the remaining activity against the corresponding enzyme control without inactivator (solid squares). The degree of acetamide incorporation by the enzyme was measured in an identical experiment, except that [^{14}C]iodoacetamide was used (open squares). The curve was the best fit to eq 1 of the inactivation data with a rate constant of 0.045 min^{-1}. (B) Concentration dependence of IA-cPLA$_2$ inactivation by iodoacetamide at pH 9.5, 37 °C, where k_{obs} is calculated from eq 1 and the line represents the best fit to eq 3 with k_{inact} = 0.2 min^{-1} and K_i = 2.5 mM.

Treatment of TNB-Labeled Enzyme with Iodoacetamide. Since DTNB and iodoacetamide both covalently inactivate cPLA$_2$ stoichiometrically, it is of interest to determine whether the two inhibitors modify the same amino acid residue. TNB-labeled IA–cPLA$_2$ was treated with iodoacetamide at pH 9.5 and 33 °C, followed by addition of DTT and assay for recovered activity. (Note that whereas the TNB-labeled enzyme is stable, yielding recoverable activity at 33 °C, no activity can be recovered from the TNB-modified enzyme after 15 min at 37 °C.) As shown in Figure 8, the free native enzyme loses most of its activity under the reaction conditions (solid circles); however, incubation of the TNB-labeled enzyme with iodoacetamide followed by DTT treatment leads to the recovery of most of the enzyme activity (solid squares). In effect, the TNB-labeled enzyme is protected from permanent inactivation by iodoacetamide. This result suggests that the two inhibitors modify either the same vulnerable cysteine, which leads to loss of enzyme activity, or distinct amino acid residues in very close proximity.

Identification of the Amino Acid Residues Labeled by Iodoacetamide. Amino acid composition analysis was performed for [^{14}C]iodoacetamide-labeled enzyme to identify

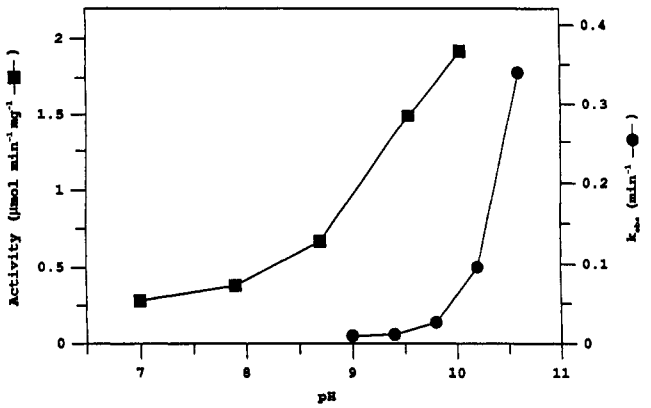


FIGURE 7: pH dependence of IA–cPLA$_2$ activity and inactivation by iodoacetamide. IA–cPLA$_2$ was assayed at several pH values, 37 °C, as described under Materials and Methods, except that 250 μM TX-100 was used in the place of 2.7 μM deoxycholate (solid squares). IA–cPLA$_2$ (0.15 mg/mL) was incubated at 37 °C with 10 mM iodoacetamide. The reaction buffer contained 100 mM glycine at various pH values, 100 mM NaCl, 1 mM $CaCl_2$, and 30% glycerol. Aliquots were removed from the reaction mixture and assayed for activity. The k_{obs} was obtained by fitting the data to eq 1 (solid circles).

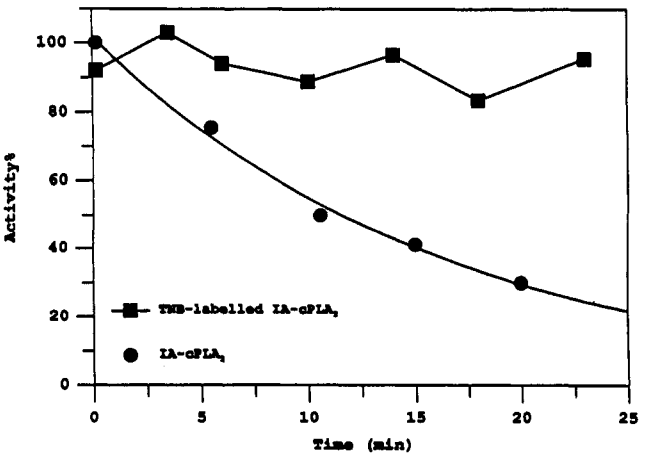


FIGURE 8: Recovery of activity after IA treatment of TNB-labeled IA–cPLA$_2$. TNB-labeled cPLA$_2$ (solid squares) or IA–cPLA$_2$ (solid circles) (0.05 mg/mL) was incubated with 10 mM iodoacetamide at pH 9.5, 33 °C. Aliquots of mixture were removed at various times, diluted into 4 volumes of 5 mM DTT, and incubated at room temperature for 20 min followed by activity determination. The curve is the best fit to eq 1 for IA–cPLA$_2$ inactivation, with a rate constant of 0.068 min^{-1}.

the type of amino acid that was modified in our experiments. IA–cPLA$_2$ was labeled by [^{14}C]iodoacetamide at pH 9.5/37 °C as described under Materials and Methods. Figure 9 displays the HPLC chromatogram of the resulting sample. The bars in the figure track radioactive eluate and indicate that ^{14}C is exclusively associated with carboxyamidomethylated cysteines (the hydrolyzed product of carboxamidomethylated cysteines), establishing that iodoacetamide at pH 9.5 modifies a cysteine residue, leading to the complete loss of enzyme activity.

Amino acid composition analysis was also carried out with cPLA$_2$, which was labeled by [^{14}C]iodoacetamide at pH 8.0/37 °C but not inactivated. The results indicate that the radioactivity was exclusively associated with cysteine residues.

To identify the labeled cysteine residue that is responsible for enzyme inactivation, [^{14}C]iodoacetamide was used to covalently modify cold IA–cPLA$_2$ under the conditions of inactivation as described under Materials and Methods. The resulting radiolabeled protein was digested with endoproteinase

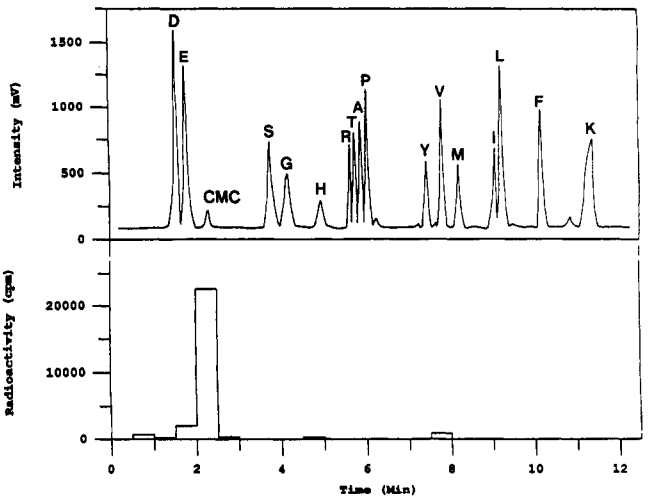


FIGURE 9: HPLC chromatogram of derivatized amino acids from cPLA$_2$ labeled with [^{14}C]iodoacetamide. IA–cPLA$_2$ was radiolabeled with [^{14}C]iodoacetamide at pH 9.5, 37 °C, and exhaustively hydrolyzed for amino acid analysis as described under Materials and Methods. The solid line is the HPLC chromatogram of the derivatized amino acids, whereas the bars represent the radioactive counts of each 0.5-min fraction from the column eluate.

Lys-C in 2 M guanidine at pH 8. Lys-C was selected because of its activity at the high concentration of guanidine (2 M) required for effective denaturation of the enzyme (Riviere et al., 1991). Figure 10 displays the HPLC chromatogram of the digested proteins and the corresponding radioactive counts associated with each peak. The majority of the radioactivity is located in peak 52 (at 42% acetonitrile). Small amounts of radioactivity are found in other peaks as well, possibly due to incomplete proteolysis or a low degree of random labeling during the enzyme inactivation.

Figure 11A shows the sequence of the isolated peptide 52 as determined with an automatic protein sequencer and the corresponding sequence derived from cDNA clone. Since all of the cysteines on the enzyme are carboxamidomethylated by iodoacetamide, the corresponding CMC standard is used to identify the hydrolyzed carboxymethylated cysteines. Peptide 52 consists of 40 amino acids, and its sequence matches exactly the sequence of Val^{319}–Lys^{358} derived from cDNA (Clark et al., 1991). Normally, endoproteinase Lys-C should cleave the peptide bond between lysine carboxyl and adjacent amino acids. Therefore, a peptide of 17 amino acids (Val^{319}–Lys^{335}) is expected. However, on the basis of the sequencing results, it appears that the peptide bond between Lys^{335} and Pro^{336} is resistant to the cleavage by Lys-C, a phenomenon which has been observed previously for such a linkage (Kurt Jarnagin, Syntex, Palo Alto, CA, personal communication).

A fraction of the sample was removed from each cycle during the sequencing of the first 20 amino acids of peptide 52 and counted for radioactivity. Figure 11B shows the plot of the radioactivity counts versus the sequenced amino acids. Fraction 6, which corresponds to Cys^{324}, is the major residue bearing radioactivity. A modest number of counts are associated with Cys^{331}, although it is only six residues away from Cys^{324}. The results in Figure 11 clearly show that Cys^{324} is a vulnerable cysteine which is modified by iodoacetamide during the inactivation leading to loss of cPLA$_2$ activity.

DISCUSSION

Modification of cPLA$_2$ by thiol modifying agents produces a variety of species depending on the conditions, as outlined in Schemes 2 and 3. As many as five amino acid residues of

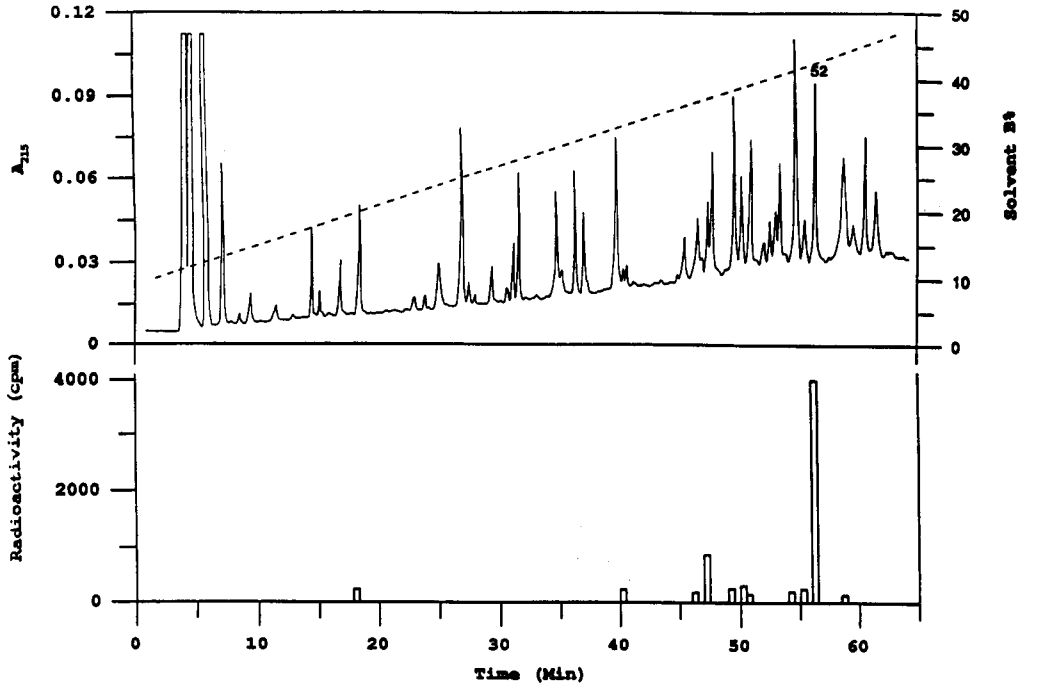


FIGURE 10: HPLC chromatogram and the corresponding radioactivity of Lys-C-digested cPLA$_2$ labeled with [^{14}C]iodoacetamide. The labeled enzyme was digested by Lys-C in 2 M guanidine hydrochloride at 37 °C, and the peptides were separated by C_{18} reversed-phase HPLC column as described under Materials and Methods. An aliquot of each peak fraction was removed and counted for radioactivity (bars).

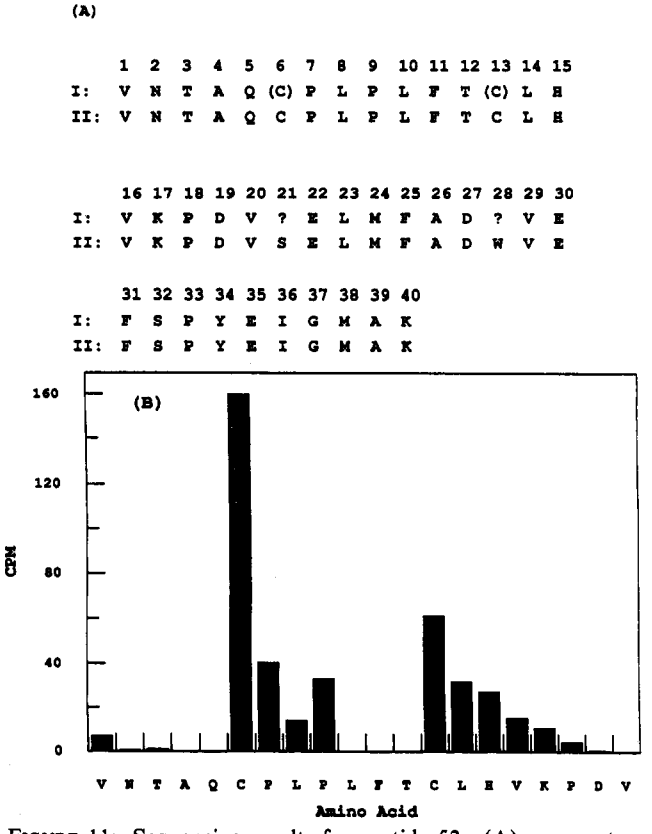
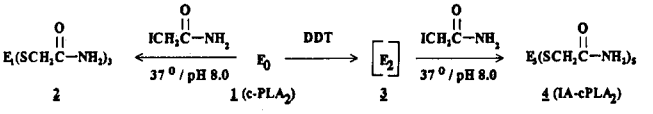


FIGURE 11: Sequencing results for peptide 52. (A) represents a comparison between the sequence of peptide 52 (I) and the corresponding sequence derived from cDNA (II). The amino acids marked by the parentheses are eluted as carboxyamidomethylated cysteines. (B) shows the ^{14}C counts of the first 20 amino acids of peptide 52.

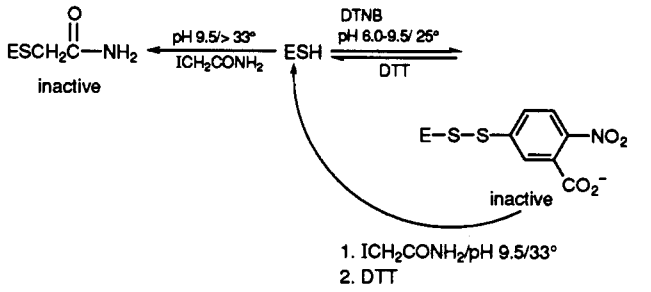
cPLA$_2$ are modified by iodoacetamide at pH 8.0 to form IA–cPLA$_2$, which is stable in the absence of DTT (Figure 1). Amino acid analysis of [^{14}C]iodoacetamide-labeled enzyme shows exclusive carboxamidomethylation of cysteine residues

Scheme 2: Chemically Modified Forms of cPLA$_2$[a]



[a] E_1, E_2, and E_3 are all modified active forms of cPLA$_2$ that are derived from treatment of cPLA$_2$ (E_0 in scheme) with chemical reagents. E_0 is cPLA$_2$ containing partially oxidized cysteine residues in assay buffer free of DTT and is up to 40% less active than E_2 or IA–cPLA$_2$. Treatment of E_0 with iodoacetamide labels three cysteine residues, giving $E_1(SCH_2CONH_2)_3$. Fully reduced enzyme (E_2) is obtained from pretreatment of E_0 with DTT, and then modified by iodoacetamide to give $E_3(SCH_2CONH_2)_5$, which we refer to as IA–cPLA$_2$ in text, and ESH in Scheme 3.

Scheme 3: Chemical Labeling of IA–cPLA$_2$ (ESH)[a]



[a] IA–cPLA$_2$ is inactivated irreversibly by 1 equiv of iodoacetamide at pH 9.5, >33 °C. Alternatively, 1 equiv of DTNB reversibly inactivates the enzyme by thiol–disulfide exchange. In the disulfide (inactive) form, the enzyme is not affected by treatment with iodoacetamide, as enzyme activity can be regenerated with DTT.

under the reaction conditions. The fact that modification of such cysteines does not affect enzyme activity indicates that they are not essential to enzyme catalysis or for maintaining the enzyme active conformation and that they may reside on the enzyme surface in free contact with solvent.

Of the five cysteine residues, two are apparently in close proximity, forming a disulfide bond under oxidative conditions as evidenced by the fact that only three cysteine residues are labeled when DTT is separated from the enzyme before it is

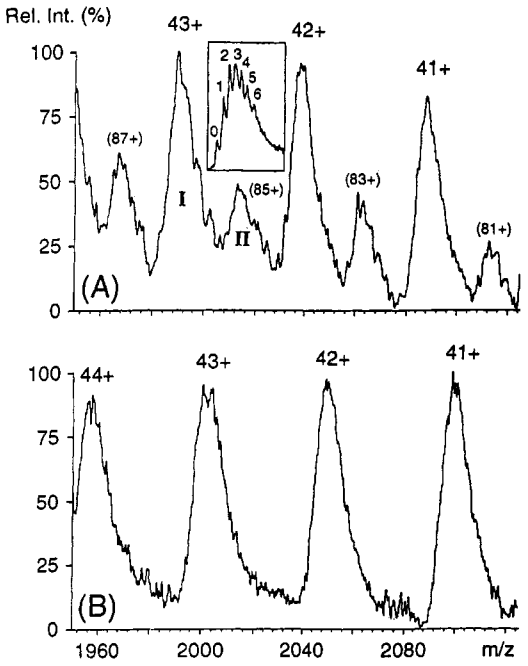


FIGURE 12: Partial electrospray mass spectrum of U937 $cPLA_2$. A triple quadrupole mass spectrometer (API III MS/MS system, Sciex, Thornhill, ON, Canada) was used for the measurements. The sample solution, containing 10% acid, 5 mM ammonium acetate, and 2.0 mg/mL enzyme, was delivered to the instrument at the rate of 1 μL/min. The charge states of 41+ to 44+ ions are shown. (A) is the spectrum of $cPLA_2$ obtained in the absence of DTT. Peaks I and II correspond to molecular masses of 85 216 and 170 000 Da, respectively. The charge states of 170 000 peaks are shown in parentheses. The inset is the high-resolution spectrum of 43+ charge-state monomer. The peaks marked 0–6 are 83 ± 3 Da apart, suggesting that they are due to different phosphorylation states of $cPLA_2$ [for $cPLA_2$ phosphorylation, see Lin et al. (1992)]. (B) is the spectrum of IA–$cPLA_2$.

treated with iodoacetamide (Figure 1, solid circles). The oxidation of these two cysteines leads to up to 40% loss of enzyme activity which can be recovered by treatment with DTT. A disulfide bond may also form intermolecularly as indicated by an MS analysis of $cPLA_2$ (Figure 12). When $cPLA_2$ is subjected to electrospray MS analysis after DTT is removed from the solution, a major peak of molecular mass 85 216 Da is observed. Also, a peak at 170 000 ± 500 Da is observed with ca. 25% of the signal intensity of the component at 85 216 Da (Figure 12A). However, the 170-kDa peak is not detected when IA–$cPLA_2$ is subjected to the same analysis (Figure 12B), indicating that dimerization is a consequence of the reaction of at least one of the cysteines that is vulnerable to carboxamidomethylation at pH 8.0.

Inactivation of IA–$cPLA_2$ by DTNB or iodoacetamide at pH 9.5 requires essentially 1 equiv of reagent (although, upon denaturation of IA–$cPLA_2$ with 4 M guanidinium chloride, four cysteines are titratible with *n*-$C_{10}H_{21}S$–TNB, accounting for a total of nine cysteines as expected from the primary amino acid sequence). Modification of a single cysteine per enzyme accounts for the loss of the enzyme activity (Figures 4 and 6). The fact that the stoichiometry of incorporation of radiolabel from [^{14}C]iodoacetamide parallels the loss of enzyme activity suggests that the inactivation of IA–$cPLA_2$ by thiol modification reagents is unlikely to be a consequence of indiscriminant labeling of enzyme residues. The reversibility of the DTNB inactivation by DTT demonstrates that the loss of enzyme activity is due to a specific, well-behaved, chemical event, likely involving a thiol–disulfide exchange reaction.

The kinetics of DTNB inactivation are somewhat complicated (Figure 2). The inactivation rate is hyperbolic at low inhibitor concentrations, while at concentrations greater than 1 mM a linear relation is observed up to 5 mM. One possible explanation for this biphasic behavior is the existence of an equilibrium between two forms of enzyme (Scheme 1).

$$k_{obs} = \frac{k_1 k_3}{k_2 + k_3}[I] + \frac{k_1'[I]}{(k_2'/k_3') + [I]} \qquad (8)$$

A series of disulfides patterned after DTNB, with one nitrobenzoate moiety replaced by an aliphatic chain, have also proven to be effective inactivators of $cPLA_2$ (Table 1). The correlation of hydrophobicity of the aliphatic chain with the rate of inactivation suggests that the reactive enzyme site has hydrophobic character. The accelerating effect of hydrophobic functionality on enzyme inactivation has been observed by us in other series as well, i.e., the maleimide derivatives (Table 2). Interestingly, immediately following enzyme inactivation with $C_{10}H_{21}S$–TNB, only 70% of the original activity could be recovered when treated with 1 mM DTT. This permanent loss of activity could be the result of interactions with the long acyl chain, a phenomenon we have observed with a variety of lipophilic reagents.

To explore the possibility that noncovalent effects related to the C_{10} chain were partially responsible for the inactivation, the corresponding thioether [4-(decanylthio)-2-nitrobenzoic acid], which cannot undergo thiol–disulfide exchange, was prepared. The latter exhibited significant inhibition (the inhibition rate was approximately one-third of the value for the $C_{10}H_{21}$–disulfide analog), which could not be reversed by DTT treatment. Thus, inactivation by long-chain DTNB analogs appears to be a complex process to which both the thiol–disulfide exchange reaction and noncovalent interactions between enzyme and the lipid acyl chain contribute. Mechanisms underlying what appears to be noncovalent inactivation may involve the irreversible displacement of key hydrophobic domains from positions in their active conformation(s) by such compounds which act like denaturing agents. Indeed, we have observed that sulfonate detergents at low micromolar concentrations and numerous compounds containing charged groups and long hydrophobes, which are unlikely to covalently label the enzyme, inhibit $cPLA_2$ at least partially irreversibly.

The pH dependencies of inactivation by iodoacetamide and DTNB exhibit a rate acceleration at high pH (>9). This observation is not unexpected since the rates of the thiol–disulfide interchange and carboxamidation by iodoacetamide are dependent upon ionized thiols, the pK_a values of which are generally in the region of 8–10. The conditions observed for inactivation by iodoacetamide are relatively severe, pH >9, T > 33 °C, which may indicate that a conformational, or other barrier, must be overcome for alkylation to occur.

For inactivation by DTNB, the pH profile shows a pK_a between 6.5 and 8, suggesting that the rate in this region is governed by deprotonation of a group with a pK_a ~7. This is consistent with a requirement for protonation by a histidine residue. Interestingly, the corresponding bis(methyl) ester of DTNB does not inactivate IA–$cPLA_2$, which may reflect preferential binding of DTNB by an enzymatic proton donor.

With respect to the immediate environment of the vulnerable cysteines, Cys^{324}, a residue that is completely modified by iodoacetamide, resides in a relatively hydrophobic region based on the primary amino acid sequence. Comparison of the amino acid sequences of the $cPLA_2$ enzymes from human, rabbit, rat, and zebra fish shows that Cys^{324} is conserved in all but the zebra fish enzyme (Ron Kriz, Genetics Institute, Cambridge, MA, personal communication). On the other hand,

Cys^{331} is conserved in all four cPLA$_2$ species and lies within a conserved region of 38 amino acids.

We have recently prepared the C324A and C331A mutants, which are active, and the C324Q and C331Q mutants, which are largely inactive, in micellar assays. On the basis of site-directed mutagenesis studies, the cysteine residues cannot be essential for catalysis. Nevertheless, they must occupy a region of the native enzyme that is vulnerable to inhibition when blocked with moieties at least as large as acetamido, which creates a (homo)glutamine-like side chain. In fact, Cudrey et al. (1993) have shown that enzyme cysteine thiols in lipases, spatially remote from the active site, can abolish enzyme activity, perhaps, by sterically hindering essential conformational changes.

In summary, a cysteine residue of cPLA$_2$ can be modified by either iodoacetamide or DTNB, resulting in stoichiometric loss of enzyme activity. Inactivation by iodoacetamide is a consequence of labeling primarily Cys^{324}. The DTNB inactivation is stoichiometric, and reversible by DTT. The TNB moiety actually protects the enzyme from permanent inactivation by iodoacetamide, suggesting that the same cysteine residue in the free enzyme (or one proximal) is being modified by DTNB and iodoacetamide.

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