Site-Directed Mutagenesis of Cys^{324} and Cys^{331} in Human Cytosolic Phospholipase A_2: Locus of Action of Thiol Modification Reagents Leading to Inactivation of $cPLA_2$

Bin Li,[‡] Ling Xia,[§] Allen Krantz,[||] and Zhengyu Yuan*,[⊥]

Syntex Incorporated, 2100 Syntex Court, Mississauga, Ontario, Canada L5N 3X4

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ABSTRACT: Human cytosolic phospholipase A_2 contains two cysteines, Cys^{324} and Cys^{331}, chemical modification of which using thiol modifying reagents abolishes the activity of the enzyme [Li et al. (1994) *Biochemistry 33,* 8594−8603]. To verify the functional importance of the two cysteine residues, site-directed mutagenesis has been used to create six mutations at positions 324 and 331. The mutant enzymes include C324A, C331A, C324Q, C331Q, C324R, and C331S. Complete loss of activity is observed for C331Q, whereas the other mutants have retained varying degrees of activity. These results show that neither Cys^{324} nor Cys^{331} is catalytically essential for the enzyme activity. Further chemical modification studies of the mutant enzymes by thiol-specific reagents suggest that modification of Cys^{331} is responsible for the complete loss of the enzyme activity. The possible roles of Cys^{324} and Cys^{331} are discussed.

Phospholipase A_2 (PLA_2)[1] catalyzes the hydrolysis of phospholipids at the *sn*-2 position to produce lysophospholipids and fatty acids. Historically, PLA_2s from secretory sources ($sPLA_2$) have been subjected to extensive studies (Dennis, 1987; Jain & Berg, 1989; Scott et al., 1990; Ramirez & Jain, 1991). Recently, attention has been focused on the intracellular, high molecular weight form of PLA_2 ($cPLA_2$), which specifically releases arachidonic acid from the corresponding lipids (Clark et al., 1990; Diez & Mong, 1990; Kramer et al., 1991).

Although both secretory PLA_2 and cytosolic PLA_2 catalyze the release of fatty acid from the *sn*-2 position of lipids, there are several major differences between these two enzymes besides their molecular weights. These include the Ca^{2+} requirement for activity, disulfide bond formation, and the substrate specificity (Yoshihara & Watanabe, 1990; Zupan et al., 1991; Wijkander & Sundler, 1992). Also, there is no amino acid sequence homology between these two forms of enzyme. The substrate specificity of $cPLA_2$ for arachidonyl-containing phospholipid is undoubtedly most significant, as the released arachidonic acid can be further metabolized to generate a number of potent inflammatory mediators (Samuelsson et al., 1987). The possibility that $cPLA_2$ catalyzes the rate-limiting step in the arachidonic acid metabolic pathway makes $cPLA_2$ a very attractive target for novel drug development.

Previously, we have described the results of chemical modification studies of $cPLA_2$, in an attempt to explore the essentiality of various types of residues of the enzyme and to provide mechanistic insight (Li et al., 1994). Through these chemical modification studies, we have identified a region of the enzyme, possibly overlapping the active site, that is vulnerable to thiol modification reagents. Both DTNB and iodoacetamide completely inactivate $cPLA_2$ in a stoichiometric manner under appropriate conditions for enzyme inactivation in which surface cysteines have been "capped". The critical amino acids being modified have been identified as Cys^{324} and Cys^{331}, both of which have been shown to be labeled by radioactive iodoacetamide. In order to verify the functional significance of the two cysteine residues and study them individually, we have carried out site-directed mutagenesis creating six mutant enzymes at positions 324 and 331. The enzyme activities, together with chemical modification results, have provided information about these two cysteine residues and also shed light on the inactivation mechanism of $cPLA_2$ by thiol modifying reagents.

MATERIALS AND METHODS

Materials. PAPC was purchased from Avanti Polar Lipids. [^{14}C]PAPC (53 mCi/mmol) was purchased from Amersham. Iodoacetamide and DTNB were obtained from Sigma. The ECL Western blot kit was purchased from Amersham. The cDNA of $cPLA_2$ inserted into the plasmid vector pMT and rabbit polyclonal antibody for $cPLA_2$ were gifts from Genetic Institute. The site-directed mutagenesis kit including pTZ plasmid was from Pharmacia.

Site-Directed Mutagenesis. The $cPLA_2$ gene from the pMT2-$cPLA_2$ plasmid was first cloned into pTZ19 phagemid, which is capable of generating single-stranded DNA with the help of helper phage M13KO7. The vector pTZ-$cPLA_2$ was constructed by subcloning the 2.8 kb *Pst*I/*Sal*I fragment carrying $cPLA_2$ cDNA from pMT-$cPLA_2$ into the corresponding sites of the phagemid pTZ19. Site-directed mutagenesis was carried out using single-stranded and uridylated DNA as template, as described by Kunkel (1985). A site-

* To whom correspondence should be addressed.

[‡] Present address: Roche Bioscience, 3401 Hillview Ave., Palo Alto, CA 94304.

[§] Present address: Sick Children's Hospital, Toronto, Ontario, Canada.

[||] Present address: RedCell, Inc., 270-B Littlefield Ave., South San Francisco, CA 94080.

[⊥] Present address: Affymax Research Institute, 4001 Miranda Ave, Palo Alto, CA 94304.

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[1] Abbreviations: CAMC, carboxamidomethylated cysteine; $cPLA_2$, cytosolic phospholipase A_2; DMEM, Dulbecco's modified essential medium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IA, iodoacetamide; PAPC, 1-palmitoyl-2-arachidonylphosphatidylcholine; [^{14}C]PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonylphosphatidylcholine; PLA_2, phospholipase A_2; TNB, 5-thio-2-nitrobenzoic acid.

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Table 1: Mutagenic Primers Used To Create Site-Directed Mutants of cPLA$_2$ [a]

Primers	Sequence
Wild Type Sequence	5'-GTT AAT ACT GCA CAA *TGC* CCT TTA-3'
	324
C324A	5'-GTT AA**C** ACT GCA CAA *GCC* CCT TTA-3'
	HpaI
C324R	5'-GTT AA**C** ACT GCA CAA *CGA* CCT TTA-3'
C324Q	5'-GTT AA**C** ACT GCA CAA *CAG* CCT TTA-3'
Wild Type Sequence	5'-CTT TTC ACC *TGT* CTT CAT GTC-3'
	331
C331A	5'-CTT TTC ACC *GCT* CT**A** CAT GTC-3'
	AflIII
C331S	5'-CTT TTC ACC *AGT* CT**A** CAT GTC-3'
C331Q	5'-CTT TTC ACC *CAG* CT**A** CAT GTC-3'

[a] Wild-type sequence is listed for comparison with the mutant primers. Positions 324 and 331 are in underlined italic. Base changes for the mutants are in boldface. A silent mutation is introduced in all the mutant primers to create an extra restriction site (underlined) for the purpose of screening.

directed mutagenesis kit from Pharmacia was used for the mutagenesis experiment. Table 1 lists the mutagenic primers used for the mutagenesis, with mutant codons in boldface. A second mutation was also introduced in each primer to create an extra restriction site for the screening purposes. These second mutation sites are silent mutations, leading to no change of amino acid sequence after expression. Mutants were initially screened by restriction digest, using the introduced restriction sites in the primers. The positive mutants were subcloned back to pMT-cPLA$_2$ plasmid for transient expression in mammalian cells. Two unique restriction sites, *Pst*I and *Eco*NI, were used for subcloning. The inserted fragments between *Pst*I and *Eco*NI in all mutant pMT-cPLA$_2$ plasmids were sequenced to confirm the mutations before mammalian cell expression. Sequencing was carried out using an automatic sequencer at the Biotechnology Center at Sentex (Palo Alto, CA).

Transient Expression of PLA$_2$ in COS Cells. Plasmid DNA containing wild-type or mutant cPLA$_2$ was used to transfect COS-1 cells by the DEAE-dextran method (Kaufman, 1990). Ten micrograms of plasmid DNA was mixed with DEAE-dextran and added to each 100 mm dish containing COS-1 cells at 90% confluency. The cells were incubated at 37 °C for 30 min. All the transfections were done in triplicate. The cells were then treated with chloroquine for 3 h and incubated with DMEM medium for 72 h at 37 °C. Harvest of cells was carried out by scraping the cells with rubber spatulas and centrifugation at 800*g* for 15 min at 4 °C. The cell pellets were resuspended in ice-cold lysis buffer containing 10 mM HEPES (pH 7.5), 30% w/v glycerol, 1 mM EDTA, 1 mM DTT, 1 μg/mL leupeptin, and 1 mM PMSF, and sonicated twice for 20 s with 30 s in between. The cell suspension was on ice during and after each sonication. The lysate was centrifuged in a table-top centrifuge (15000*g*). The supernatant was used for Western blot analysis and enzyme activity assay. A second centrifugation at 100000*g* was carried out to obtain supernatant which was used for inactivation studies. Both the lysates and supernatants were stored at −80 °C.

Western Blot Analysis. For all mutants, equal volumes of lysate supernatant were subjected to SDS−PAGE analysis using the Phast System from Pharmacia and then transferred to nitrocellulose paper by blotting at 70 °C for 1 h. Rabbit polyclonal antibody against cPLA$_2$ was used to detect human cPLA$_2$ enzyme. The blot was visualized using the ECL system from Amersham. The final images were analyzed by a PDI scanning densitometer (PDI, Huntington Station, NY) to quantitate the protein bands. Intensities of the mutant proteins were calculated relative to that of the wild type (100%).

Enzyme Assays. Enzyme activities were determined in a mixed micelle assay using [^{14}C]PAPC as substrate as described previously (Li et al., 1994). Supernatants of the cell lysates were used directly to assay PLA$_2$ activity without further purification. Cell lysate supernatant (10 μL) was mixed with 100 μL of substrate mixture containing 80 mM glycine (pH 9.0), 1 mM $CaCl_2$, 2.67 mM deoxycholate, and 70% w/v glycerol, and then incubated at 37 °C for 5 min. The reaction mixture was quenched with 200 μL of the stop solution (70:30:1 mixture of hexane/1,4-dioxane/acetic acid) and processed using solid phase extraction columns. The blank control used the lysate supernatant of COS cells transfected with pMT2 plasmid vector without cPLA$_2$ gene.

Inactivation of cPLA$_2$. Inactivation of cPLA$_2$ by iodoacetamide was carried out in 100 mM glycine (pH 9.5), 100 mM NaCl, 10 mM $CaCl_2$, and 30% w/v glycerol at 37 °C as described before (Li et al., 1994). Supernatant (20 μL) of the wild-type or mutant enzyme cell lysates was mixed with 80 μL of iodoacetamide to give a final concentration of 5 mM, and then incubated at 37 °C. At various time intervals, enzyme activity was determined by assaying 10 μL of the enzyme/IA reaction mixtures using the mixed micelle assay as described above. The control was performed with the enzyme but without iodoacetamide.

Inactivation by DTNB was carried out in 50 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM $CaCl_2$, and 30% w/v glycerol at 37 °C. Supernatants of the cell lysates were mixed with DTNB to give a final concentration of 6 mM DTNB. Aliquots were taken out at various time intervals to assay the enzyme activity. A control without DTNB was performed for each inactivation experiment.

Alternatively, supernatants were first treated under noninactivating conditions (Li et al., 1994) with 10 mM iodoacetamide in 50 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM $CaCl_2$, and 30% w/v glycerol at 37 °C for 60 min, and then mixed with DTNB to give a final concentration of 6 mM DTNB and assayed at various time intervals for the enzyme activity.

RESULTS

Site-Directed Mutagenesis of Cys^{324} and Cys^{331}. Six mutations were created at positions 324 and 331 of cPLA$_2$ using single-stranded and uridylated DNA as template. Because the selection is based on uridylation of the single-stranded template DNA and the subsequent inactivation of the uracil-containing template strand, replication of the second strand DNA containing mutagenic primer leads to high yield of mutagenesis. Initial screening by restriction digestion showed that there were positive mutations among six picked colonies for each mutant. Specifically, the frequency of positive mutations is as follows: C324A, 3/6

Table 2: Enzyme Activities of the COS-Expressed Wild-Type and Mutant $cPLA_2$s in Cell Lysate Supernatants

enzyme[a]	activity[b] (pmol min^{-1})	[cPLA2][c]	relative specificity (%)[d]
wild type	7.8 ± 0.3	100	100
C324A	9.5 ± 2.0	81	150
C324R	0.95 ± 0.17	70	17
C324Q	4.2 ± 1.4	132	41
C331A	9.8 ± 0.3	84	149
C331S	5.6 ± 0.6	149	48
C331Q	0.084 ± 0.028	48	2

[a] Plasmid containing each mutant was used to transfect COS-1 cells, lysed, centrifuged, and assayed for enzyme activities. [b] Assays were carried out at pH 9.0/33 °C using 10 μL of cell lysate supernatant and 100 μL of [^{14}C]PAPC substrate as described under Materials and Methods. Control contains plasmid DNA without $cPLA_2$ gene and showed no activity. Activities in the table are expressed as the amount of product produced per minute and are the average of two independent experiments. The total protein concentrations in the lysates are about 5.6 mg/mL. [c] $cPLA_2$ concentrations were determined from Western blot using a scanning densitometer as described under Materials and Methods and expressed as relative to the wild type (100%). [d] The data in this column are the enzyme activities divided by $cPLA_2$ concentrations as expressed in percentage.

(three positives out of six colonies); C331A, 4/6; C324Q, 3/6; C324R, 6/6; C331Q, 4/6; and C331S, 5/6. All mutations were confirmed by DNA sequencing which showed no extra mutations within the region that was inserted back to the wild-type $cPLA_2$ gene in the expression vector, pMT-$cPLA_2$.

Transient Expression of Mutant $cPLA_2$ Genes in COS Cells. Plasmid DNA containing $cPLA_2$ genes was used to transiently transfect COS-1 cells. The wild-type $cPLA_2$ gene was used as a control and expressed along with the mutant genes under identical conditions. Western blot analysis showed that both the wild-type and mutant genes were expressed at varying levels by COS cells. The level of mutant protein expressed as compared to that of the wild type was determined by densitometric scanning of the Western blot. The results are summarized in Table 2. Considering the possible variation of the protein expression system, the level of each mutant protein expressed in this system can be regarded to be relatively similar, suggesting that the point mutation introduced at Cys^{324} and Cys^{331} did not significantly change the protein stability.

Enzyme Activities of the Expressed Wild-Type and Mutant $cPLA_2$. Table 2 summarizes the relative enzyme activities of equal volumes of mutant lysate as compared to that of the wild-type PLA_2. Since the $cPLA_2$ concentrations of the lysate vary from each other, relative specific activities (after correction for protein concentration) of each lysate were calculated as in the fourth column in Table 2.

Changing cysteine to alanine at position 324 or 331 resulted in higher activity than the wild type: C324A, 150%; and C331A, 149%. Replacement of the cysteine at 331 with serine gave a mutant enzyme with about half of the specific activity: C331S, 48%. Substitution of cysteine with glutamine at position 331 probably completely inactivated the enzyme, whereas the protein with the same substitution at position 324 retained 41% of the activity. Arginine substitution at position 324 gave a mutant with significant loss of activity, being 17% as compared to the wild type.

Inactivation of the Mutant Enzymes by Iodoacetamide. Chemical modification by iodoacetamide under the conditions of inactivation for wild-type $cPLA_2$ was carried out

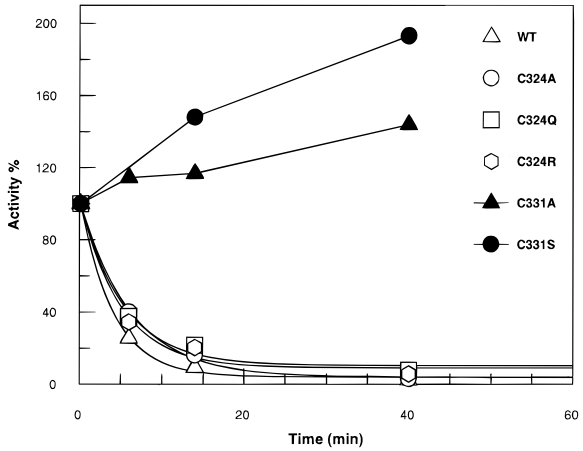


FIGURE 1: Time course of iodoacetamide inactivation of the wild-type and mutant $cPLA_2$. The experiment was carried out at pH 9.5 and 37 °C as described under Materials and Methods. Supernatants of cell lysates after sonication and centrifugation at 100000*g* were mixed with iodoacetamide to a final concentration of 5 mM, and the mixture was assayed at various time intervals. Each $cPLA_2$ had a control without iodoacetamide. Activity is expressed as a percentage of control. Curves for WT, C324A, C324R, and C324Q are from fitting to a single-exponential decay.

Table 3: Iodoacetamide Inactivation of the Wild-Type and Mutant $cPLA_2$s

	k_{inact} (min^{-1})[b]	
enzyme[a]	IA	DTNB
wild type	0.25 ± 0.03	0.26 ± 0.02
C324A	0.16 ± 0.01	0.18 ± 0.01
C324R	0.20 ± 0.05	ND[d]
C324Q	0.19 ± 0.04	ND
C331A	0	0.15 ± 0.01[c]
C331S	0	ND

[a] The activities were determined from supernatant after centrifugation at 100000*g*. [b] The rate of inactivation and corresponding errors were obtained by fitting the data to the exponential decrease of enzyme activity with time. [c] The number was obtained by fitting the data to a single-exponential decay with an offset value of 0.5. The time course in Figure 2 showed an initial loss of activity, but the activity leveled off at ~50% after 20 min. [d] ND, not determined.

for mutants C324A, C331A, C324R, C331S, and C324Q. As described previously, inactivation by iodoacetamide was observed for the wild-type $cPLA_2$ at relatively high pH (~9), leading to the complete loss of enzyme activity and stoichiometric labeling by [^{14}C]iodoacetamide. In the current experiment, supernatants from the cell lysates were used for the inactivation by iodoacetamide under such inactivation conditions. Figure 1 shows the time courses of the inactivation by iodoacetamide. The remaining activity is fitted to a single exponential equation to obtain the rate constants for inactivation, which are summarized in Table 3. Those enzymes with mutations at position 324 without changes at Cys^{331}, namely, C324A, C324Q, and C324Q, were inactivated by iodoacetamide under the same conditions that led to inactivation for the wild-type enzyme, though at slower rates. By contrast, the two mutants of Cys^{331}, C331A and C331S, showed no significant inactivation by iodoacetamide, suggesting that the modification of Cys^{331} by iodoacetamide is responsible for the activity loss of the wild-type enzyme.

Inactivation of the Mutant Enzymes by DTNB. Inactivation of the mutant enzymes C324A and C331A by DTNB was performed, and the results were compared with that of the wild-type enzyme. Our previous results on the wild-

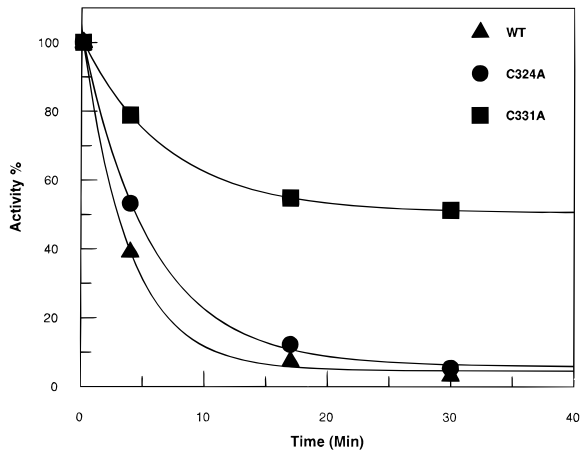


FIGURE 2: Time courses of DTNB inactivation of the wild-type, C324A, and C331A cPLA$_2$. The inactivation was performed at pH 7.5 and 37 °C as described under Materials and Methods. Supernatants of cell lysates were used for the experiment. Final DTNB concentration was 6 mM. All three curves are fitted to a single-exponential decay with offsets.

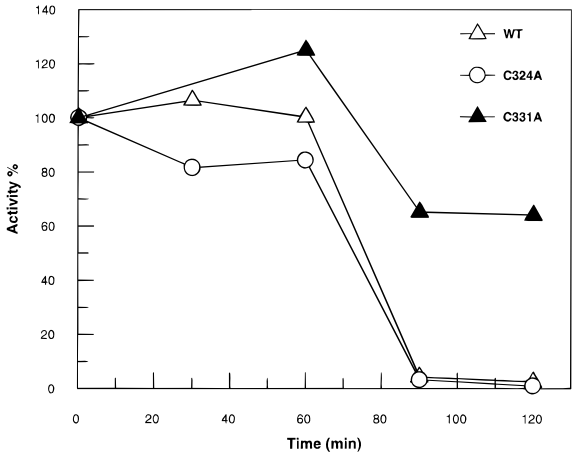


FIGURE 3: Time courses for inactivation of the wild-type, C324A, and C331A cPLA$_2$ with iodoacetamide and DTNB. The experiment was carried out at pH 7.5 and 37 °C as described under Materials and Methods. The wild-type and mutant cPLA$_2$ were incubated with 10 mM iodoacetamide for 60 min. DTNB was then added to give a final concentration of 6 mM. A control without iodoacetamide and DTNB was performed for each enzyme, and activity was expressed as a percentage of control activity.

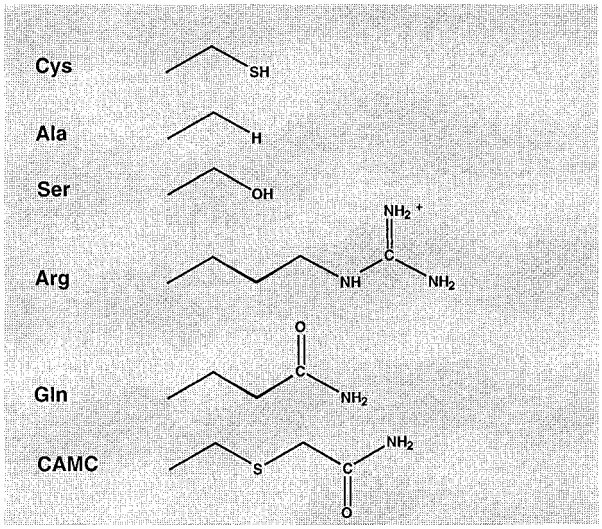
type cPLA$_2$ showed that DTNB could completely inactivate the enzyme and result in the reversible covalent addition of a single TNB residue. Figure 2 shows the time courses of the DTNB inactivation as compared with the control lacking the inactivator. The rate constants, obtained from data fitting to a single exponential decay, are presented in Table 3. The results clearly show that the wild type and C324A can be completely inactivated by DTNB. These results agree well with the inactivation results by iodoacetamide. The result for C331A is interesting in that there is an initial loss of activity, possibly due to inactivation, followed by a plateau, indicating that no further inactivation occurred. This time course suggests that when Cys^{331} is no longer available for DTNB modification (C331A), at least 50% enzyme activity is resistant to DTNB inactivation.

Treatment of the Wild-Type and Mutant Enzymes with Iodoacetamide followed by DTNB Inactivation at pH 7.5. We have observed previously that a number of thiols can be modified without significant loss of the enzyme activity. These thiols are presumably located on the surface or are accessible to iodoacetamide. The modification experiment using iodoacetamide at pH 7.5 followed by DTNB was performed on C324A and C331A to demonstrate that the vulnerable cysteine residues are not accessible to iodoacetamide at low pH (<pH 8). Incubation of the wild type as well as the mutant enzymes was carried out at pH 7.5 and 37 °C for 1 h, followed by addition of DTNB. As indicated in Figure 3, when only iodoacetamide was present at pH 7.5, no significant inactivation was observed for either mutant for up to 1 h. When DTNB was added, both wild type and C324A were inactivated, whereas C331A was only partially inactivated and the activity of the mutant enzyme leveled off at about 65% as compared to that of the control. The results here are consistent with those presented above for inactivation by DTNB and, furthermore, support the assumption that the thiol groups of Cys^{324} and Cys^{331} are not accessible at pH 7.5.

DISCUSSION

Two cysteine residues, Cys^{324} and Cys^{331}, of human cytosolic phospholipase A$_2$ are vulnerable to thiol modification reagents such as DTNB and iodoacetamide as shown in our previous studies (Li et al., 1994). To further study the functional importance of these two cysteine residues, we have made several mutations at these two positions, transiently expressed the mutant cDNA in COS cells, and studied the corresponding enzyme activities and their modifications by iodoacetamide and DTNB.

Scheme 1: Side Chains of Amino Acids and Modified Cys (CAMC)



Scheme 1 shows the side chains of cysteine and those of the mutants, as well as the iodoacetamide-modified cysteine side chain, carboxamidomethylated cysteine (CAMC). The alanine mutants were created to probe whether the thiol group in cysteine is functionally critical to catalysis. In this cysteine to alanine mutation, the thiol of cysteine is replaced by hydrogen, resulting in a side chain which lacks potential chemical reactivity and polar interactions. The cysteine to serine mutation is a conservative change: thiol for hydroxyl. Many experiments have shown that this mutation will retain to some degree chemical interactions of the thiol group. The cysteine to arginine mutant introduces a bulky and positively charged group within the mutant side chain. The glutamine

side chain closely resembles that of cysteine modified by iodoacetamide, i.e., carboxamidomethylated cysteine.

As shown in Table 2, the enzyme activities of the C324A and C331A mutants clearly indicated that neither Cys^{324} nor Cys^{331} is catalytically important for enzyme function. Alanine mutations at both positions did not affect enzyme activity at all. Since the replacement of the thiol group of a cysteine side chain with hydrogen should completely abolish the residues' ability to participate in enzyme catalysis, it is clear from these results that the thiols of Cys^{324} and Cys^{331} do not play any obvious roles of thiols in the catalysis or substrate binding.

When Cys^{324} was substituted with glutamine, 41% activity was retained, indicating that modification of Cys^{324} by iodoacetamide under inactivation conditions is not responsible for the complete loss of enzyme activity. Furthermore, even when substituted with a positively charged arginine group, C324R still retains 17% activity, thus supporting the notion that Cys^{324} is not the critical residue at the active site responsible for the iodoacetamide inactivation effect. These results indicate that modification of the Cys^{324} to a bulky entity affects substrate binding or changes the local conformation to the extent that enzyme activity is only partially lost, suggesting that Cys^{324} is not in a critical position for substrate binding.

In contrast to Cys^{324}, Cys^{331} may be in a critical position in the substrate binding pocket or may play a role in maintenance of active site integrity. When the cysteine side chain was changed to glutamine, the resultant enzyme had no activity. The iodoacetamide-modified cysteine group has a side chain that resembles that of glutamine but is longer in length, and it is probable that the glutamine mutant mimics the iodoacetamide-modified thiol group. This complete loss of enzyme activity of the C331Q mutant also agrees well with the chemical modification results, showing that modification of a single cysteine residue inactivates the enzyme. From the results on the Cys to Gln mutants, it is clear that iodoacetamide modification of Cys^{331} rather than Cys^{324} is responsible for the complete loss of enzyme activity.

To further probe the inactivation mechanism of $cPLA_2$ by thiol modification reagents, mutant forms of $cPLA_2$ were subjected to iodoacetamide and/or DTNB modification. When reacted with iodoacetamide, all three mutants at position 324 (C324A, C324R, and C324Q) have comparable inactivation rates as compared to the wild type, implying that inactivation by iodoacetamide is not the direct result of modification of Cys^{324}. In contrast to the Cys^{324} mutants, the mutants at position 331 (C331A and C331S) are resistant to iodoacetamide inactivation. Since iodoacetamide does not react with alanine or serine under these conditions, the side chains of residue 331 in C331A and C331S mutants remain intact during the modifications. These data support the notion that it is the modification of Cys^{331} that leads to the loss of enzyme activity. This provides a consistent rationale for our finding that C331Q, whose side chain resembles carboxamidomethylated cysteine, has no enzyme activity at all.

Chemical modification of $cPLA_2$ by DTNB also indicates that Cys^{331} is the residue which is primarily responsible for the enzyme inactivation by thiol modifying reagents. When treated with DTNB, C324A is inactivated in the same pattern as the wild type, though at a slower rate, whereas C331A had no significant loss of activity. These results agree well with those of the inactivation by iodoacetamide, supporting our previous conclusion that both DTNB and iodoacetamide modify the same thiol residue.

Although Cys^{331} is not catalytically important to the enzyme reaction, it is very likely that this amino acid is in a critical position in the active center or the substrate binding channel. Modification of this cysteine by DTNB or iodoacetamide results in a spacially more demanding side chain, which may prevent substrate binding. Alternatively, Cys^{331} may be in a very critical position enabling the enzyme to maintain its correct conformation. In such a case, although clearly the enzyme can fold into a stable conformation, the length of the side chain may disrupt the enzyme structure and render it inactive. The data from this study do not rule out either of these possibilities.

Modification of Cys^{324} by DTNB may partially contribute to the loss of enzyme activity, as is evident by the initial loss of activity when C331A was modified by DTNB. The remaining 50% of activity is resistant to further thiol modification. Modification of cysteine by DTNB results in a thiol nitrobenzoate group attached to the cysteine residue through a disulfide bond. The bulky side chain of DTNB-modified Cys^{324} may be responsible for the partial loss of enzyme activity.

Sequence comparison among $cPLA_2$ cDNAs from various species also points out the importance of Cys^{331} (James Clark, Genetics Institute, Inc., personal communication). Positions 326−363 (number of human $cPLA_2$) are completely conserved in $cPLA_2$s from all four species, while position 324 is in a region which varies from species to species:

	324	331
human	NTAQ**C**PLPLFT**C**LHVKPDVSELEFADWVEFSPYEIGMAKYGTFM	
murine	NAAR**C**PLPLFT**C**LHVKPDVSELEFADWVEFSPYEIGMAKYGTFM	
chicken	SEAQ**C**ALPLFT**C**LHVKPDVSELEFADWVEFSPYEIGMAKYGTFM	
zebra fish	NEGQ**C**PLPLFT**C**LHVKPDVSELEFADWVEFSPYEIGMAKYGTFM	

The sequence data, together with the results presented in this study, suggest that Cys^{331} is in a very sensitive region of the enzyme, probably in or near the enzyme active center.

It is worth noting that there are several other enzymes which can also be inhibited stoichiometrically by sulfhydryl-sensitive reagents, but the corresponding cysteine residue is not catalytically essential for enzyme activity (HMGCoA reductase: Jordan-Starck & Rodwell, 1989; lecithin−cholesterol acyltransferase: Francone & Fielding, 1991; and phosphomanose isomerase: T. N. C. Wells, Glaxo Institue for Molecular Biology, personal communication). It is not clear whether there is a common role for all these cysteine residues in these different enzymes.

In conclusion, this study differentiates between the role of Cys^{324} and Cys^{331} in the inactivation of $cPLA_2$ by thiol modifying reagents. We have found that neither residue is catalytically essential. Modification of Cys^{331} is responsible for the inactivation by thiol-specific reagents. The results also suggest that Cys^{331} is likely located in a vulnerable position on the enzyme which, when modified chemically or by site-directed mutagenesis, leads to abrogation of the enzyme activity.

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