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Identification of Essential Residues for the Catalytic Function of 85-kDa Cytosolic Phospholipase A_2

PROBING THE ROLE OF HISTIDINE, ASPARTIC ACID, CYSTEINE, AND ARGININE*

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Cytosolic phospholipase A_2 ($cPLA_2$) hydrolyzes the *sn*-2-acyl ester bond of phospholipids and shows a preference for arachidonic acid-containing substrates. We found previously that Ser-228 is essential for enzyme activity and is likely to function as a nucleophile in the catalytic center of the enzyme (Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P. L., Sportsman, J. R., Becker, G. W., Kang, L. H., Roberts, E. F., and Kramer, R. M. (1991) *J. Biol. Chem.* 266, 14850–14853). $cPLA_2$ contains a catalytic aspartic acid motif common to the subtilisin family of serine proteases. Substitution within this motif of Ala for Asp-549 completely inactivated the enzyme, and substitutions with either glutamic acid or asparagine reduced activity 2000- and 300-fold, respectively. Additionally, using mutants with cysteine replaced by alanine, we found that Cys-331 is responsible for the enzyme's sensitivity to *N*-ethylmaleimide. Surprisingly, substituting alanine for any of the 19 histidines did not produce inactive enzyme, demonstrating that a classical serine-histidine-aspartate mechanism does not operate in this hydrolase. We found that substituting alanine or histidine for Arg-200 did produce inactive enzyme, while substituting lysine reduced activity 200-fold. Results obtained with the lysine mutant (R200K) and a coumarin ester substrate suggest no specific interaction between Arg-200 and the phosphoryl group of the phospholipid substrate. Arg-200, Ser-228, and Asp-549 are conserved in $cPLA_2$ from six species and also in four nonmammalian phospholipase B enzymes. Our results, supported by circular dichroism, provide evidence that Asp-549 and Arg-200 are critical to the enzyme's function and suggest that the $cPLA_2$ catalytic center is novel.

Cytosolic phospholipase A_2 ($cPLA_2$)[1] is an 85-kDa enzyme increasingly believed to accelerate inflammation through its selective release of arachidonic acid from cellular phospholipids in response to receptor-mediated cellular signaling events (1). It is a carboxylesterase whose preferred phospholipid substrate has arachidonic acid esterified at the *sn*-2 position (1, 2), but it is also capable of hydrolyzing lysophospholipids (3, 4) and other esters (5). Its primary sequence (6, 7) bears no obvious relationship to that of the well studied 14-kDa secreted PLA_2 enzymes (8). While these two classes of PLA_2 catalyze the same reaction on the same substrates, admittedly with different preferences (1, 2), their mechanism of action appears to be different. While a tightly bound calcium ion is essential for catalysis in 14-kDa PLA_2 (9), calcium is necessary for $cPLA_2$ only for enhanced binding to its aggregated phospholipid substrates (4, 10). Much evidence supports the currently accepted model that the secreted PLA_2 enzymes have a catalytic center that lacks a serine residue and in which a tightly bound water molecule serves as the nucleophile (9, 11). In contrast, in $cPLA_2$, a serine residue (Ser-228) is essential and is likely to play this role (12, 13), as was also found recently for another cytosolic enzyme with PLA_2 activity, platelet-activating factor acetylhydrolase (14). This suggests, along with earlier evidence (4), the formation of an acyl-enzyme intermediate (arachidonoylserine 228), for which additional indirect evidence was recently obtained by examination of the comparative rates of secondary transfer of the arachidonoyl group to glycerol or to water (15).

It might be anticipated that the catalytic function of Ser-228 would be supported by a hydrogen-bonding network similar to the ones identified in other lipases (16–20) by three-dimensional structure supported by other data. We report here that, in addition to Ser-228, Asp-549 and Arg-200 are also essential for catalysis and that Cys-331, while not essential, is responsible for the sensitivity of $cPLA_2$ to NEM. The role of Arg-200 is discussed. Furthermore, we show that none of the 19 histidines in human $cPLA_2$ is essential. Therefore, histidine cannot play the central role that it plays in more conventional catalytic centers, and it is likely that $cPLA_2$ acts through a novel catalytic mechanism for acylhydrolases.

MATERIALS AND METHODS

Preparation of $cPLA_2$—Two systems were used to produce both wild-type and mutant $cPLA_2$ enzymes for analysis. For more rapid preliminary analysis, we produced $cPLA_2$ in COS-1 cells using the plasmid pCDCPT. For production of larger amounts of $cPLA_2$, we used baculovirus in insect cells with plasmid pVLCP (21). Many of the mutants

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This paper is dedicated to the memory of Professor David Gordon Sharp (1909–1994).

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[1] The abbreviations used are: $cPLA_2$, 85-kDa cytosolic phospholipase A_2; PLA_2, phospholipase A_2; NEM, *N*-ethylmaleimide; ELISA, enzyme-linked immunosorbent assay; 7-HCA, 7-hydroxycoumarinyl arachidonate; OSPC, 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CaLB, calcium/lipid-binding region of $cPLA_2$.

were analyzed in crude lysates of COS-1 or insect cells, prepared as described (12).

Construction of pCDCPT—The cPLA$_2$ coding sequence contained within the *Hin*dIII-*Eco*RV fragment of pPSJ151 (21) was ligated into M13mp18, creating M18CP, in which the *Eco*RI site within the cPLA$_2$ coding sequence was removed by site-directed mutagenesis. The resulting 2.5-kilobase *Eco*RI-*Bgl*II fragment from M18CP was ligated into pALTER (Promega) with a *Bgl*II-*Spe*I-*Sph*I linker, making pALTER-CPT. The *Eco*RI-*Spe*I fragment of pALTERCPT was used to replace the *Eco*RI-*Spe*I fragment of pCDCPFS, creating pCDCPT. pCDCPFS was constructed by inserting the entire coding sequence contained within the *Kpn*I-*Spe*I fragment of pHDCPFS (12) into pCD-PS (22).

Site-directed Mutagenesis—Two methods were used for mutagenesis of cPLA$_2$. For some of the histidine mutants (H259A, H333A, H385A, H426A, H488A, H544A, and H637A), we used a mutagenic primer containing the histidine mutation and a synthetic primer containing one of the two *Nco*I sites in cPLA$_2$ cDNA to amplify a mutagenic "megaprimer" (23). This megaprimer was then used with a synthetic primer containing the other *Nco*I site to amplify the mutant 1.53-kilobase *Nco*I fragment. This *Nco*I fragment was ligated into the baculoviral transfer vector pVLCP (21). For the other mutants, we used the Altered Sites mutagenesis system (Promega) (24). The mutagenized coding sequence was used to replace the wild-type coding sequence of either the baculoviral transfer vector pVLCP or the mammalian expression vector pCDCPT. For each point mutation, a "silent" restriction site was introduced near the mutagenic site for identification of the mutant plasmid. All mutations were confirmed by DNA sequencing. For the histidine mutants expressed in the baculovirus system, a region of the recombinant viral DNA was amplified and sequenced to confirm that the correct mutation was present after recombination with the viral genome.

For those mutations that produced inactive enzyme, it was necessary to prove rigorously that no unintended mutation might be responsible for the loss of activity. For all six mutations at Arg-200 and Asp-549, revertants were prepared, and it was demonstrated that these revertants regained full activity. This was considered preferable to sequence analysis since the reversion demonstrates a dramatic recovery of several orders of magnitude of enzyme activity. Any additional mutation that may be present and escape detection when sequencing the 2.2-kilobase cPLA$_2$ coding region is thereby ruled out as causing the loss of activity.

Detection of cPLA$_2$ by Enzyme Activity Assays, Immunoblotting, and ELISA—PLA$_2$ activity was assayed using as substrate 3 μM sonicated mixed phosphatidylcholine/dioleoylglycerol vesicles at a molar ratio of 2:1 as described (12). Immunoblotting analysis was performed on all mutant forms to confirm electrophoretic mobility equivalent to wild-type cPLA$_2$ and to estimate qualitative yield. Cell extracts were subjected to SDS-polyacrylamide gel electrophoresis/immunoblotting with M3-1/horseradish peroxidase-conjugated anti-cPLA$_2$ monoclonal antibody and the ECL detection system (Amersham Corp.) (12).

Enzyme assay data were normalized to protein content with ELISA for crude lysates or with the Coomassie Plus protein assay (Pierce) for purified enzyme. ELISA was performed as described (12) using the M12 monoclonal antibody as the capture agent and either the M3−1 monoclonal antibody or the anti-cPLA$_2$ polyclonal antibody as the detecting antibody. The specific activity values were adjusted by subtraction of background values obtained from insect cells that had not been infected with baculovirus. As more data were collected, it became apparent that some of the values for inactive mutants were slightly below those of uninfected cells, yielding negative values for the most inactive mutants, as reported in Table I. This is probably due to a slight suppression we observed of the small insect cell background activity, caused by growth of baculovirus. This suppression was detected after much of our data were collected by using baculovirus carrying no cPLA$_2$ gene. All reported specific activity values were derived from at least three independently prepared transfected COS-1 or infected Sf9 insect cell cultures.

Treatment of cPLA$_2$ with Thiol-modifying Reagents—Enzyme activity was measured as described (12), after treatment and dilution as described in the legend to Fig. 3, except that the assay was performed at 40 °C and no dithiothreitol was present. The dilution after treatment was 200-fold into the same buffer without modifying reagents, resulting in assay of 0.25 ng of enzyme.

Purification of cPLA$_2$—For measurement of circular dichroic spectra, fluorescent assay, and precise determination of enzyme-specific activities, cPLA$_2$ was purified as described (12, 25).

Analysis of Conformation by CD Spectroscopy—Far-UV CD spectra were obtained on an Aviv 61DS spectrometer. The concentration of wild-type and mutant cPLA$_2$ enzymes was in the range of 0.1–0.25 mg/ml in solutions containing 50 mM phosphate, 100 mM NaCl, and 20 μM dithiothreitol adjusted to pH 7.5. Spectra were recorded at 25 ± 1 °C using cylindrical quartz cuvettes with path lengths of 0.05 and 0.1 cm. For each sample, three separate spectra were collected and averaged using a step interval of 0.5 nm and an average time of 5 s. The protein spectra were corrected by subtracting a blank solution containing 50 mM phosphate and 100 mM NaCl adjusted to pH 7.5. The final results are reported as mean residue ellipticity in units of degrees·cm^2·dmol^{-1} calculated using a mean residue weight of 113.9.

Preparation of 7-Hydroxycoumarinyl Arachidonate (7-HCA)—To a solution of arachidonic acid (943 mg, 3.10 mmol) in methylene chloride (22 ml) was added 7-hydroxycoumarin (557 mg, 3.44 mmol), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (1.618 g, 3.11 mmol), and diisopropylethylamine (1.1 ml, 6.31 mmol) in that order. The reaction was stirred at room temperature overnight. The reaction was diluted with methylene chloride (30 ml) and washed with saturated aqueous potassium dihydrogen phosphate (3 × 10 ml). The organic layer was dried over $MgSO_4$, filtered, and concentrated to give an oil. The crude material was purified by flash column chromatography, eluting with 1) 5% EtOAc/hexanes and 2) 10% EtOAc/hexanes, to give 7-hydroxycoumarinyl arachidonate as a colorless oil (948 mg, 2.11 mmol) in 68% yield. The ^{1}H NMR data for this compound are identical to those reported in Ref. 5 (mass spectrometry, *m/z* 448).

Fluorescence Assays with 7-HCA—7-HCA was prepared in the form of sonicated mixed vesicles with OSPC (Avanti Polar Lipids) as described (5). The fluorescence enzyme assay was initiated by addition of cPLA$_2$ or its R200K mutant into the stirring liposome suspension in a 3-ml plastic cuvette (Evergreen, Pico Rivera, CA). Fluorescence of the hydrolysis product, 7-hydroxycoumarin, was followed in an SLM 48000 spectrophotofluorometer (SLM-AMINCO, Urbana, IL) with excitation at 360 nm and emission 460 nm. Data were collected and stored for 200 s at 1-s intervals.

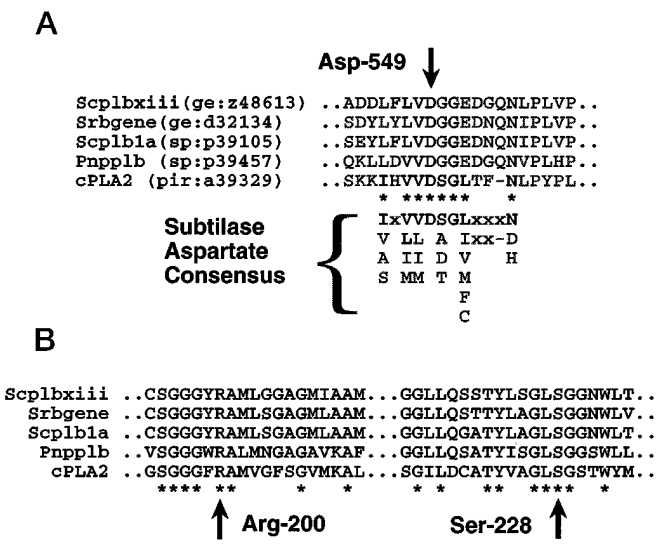

FIG. 1. **Alignment of catalytic residues of human cPLA$_2$ with subtilisin family consensus sequences and with phospholipase B proteins.** *Arrows* show the positions of the cPLA$_2$ catalytic residues. *A*, the subtilisin aspartic acid motif (16) is shown with its allowable substitutions to the right of the *brace*. The common motif elements in cPLA$_2$ and several phospholipase B proteins align the catalytic aspartate with Asp-549 of cPLA$_2$. *Asterisks* show the consensus positions matched by cPLA$_2$. The accession numbers of the sequences are shown in parentheses (*ge:*, GenBank™/EMBL; *sp:*, Swissprot; *pir:*, Protein Identification Resource). *Dashes* indicate variable spacing in the motif pattern and corresponding adjustment to align cPLA$_2$. *B*, regions surrounding Arg-200 and Ser-228 (*arrows*) for the same set of proteins. *Asterisks* show totally conserved residues.

RESULTS

Aspartate—A motif was found within the cPLA$_2$ sequence (Fig. 1*A*) that includes Asp-549 and perfectly matches the specifications for the catalytic aspartic acid residue in the subtilisin family of proteolytic enzymes (26). The motif was also aligned

TABLE I
Specific activities of wild-type cPLA$_2$ and cPLA$_2$ mutated at Asp-549 and Arg-200, produced in baculovirus-infected insect cells

The enzyme activity was normalized by ELISA for crude lysates (unmarked) and by quantitative protein assay for the indicated purified mutant enzymes. Background values from uninfected cells were subtracted as described under "Materials and Methods."

Mutation	cPLA$_2$-specific activity
	nmol/min/mg
cPLA$_2$	2700 ± 500
D549A	−0.36 ± 0.06
D549N	0.75 ± 0.05
D549E	0.14 ± 0.04
D549N (purified)	8.5
D549E (purified)	1.3
R200A	−0.16 ± 0.05
R200H	−0.27 ± 0.04
R200K	6.4 ± 0.3
R200K (purified)	14

with imperfect but good matches in phospholipase B from *Penicillium* and yeast (Fig. 1*A*). When Asp-549 was mutated to alanine, all activity was lost; however, low but measurable activity remained when Asp-549 was substituted with either glutamic acid or asparagine (Table I). To confirm that no additional unintended mutation might have caused the drastic loss of activity, each of these three mutations was reverted to wild-type cPLA$_2$, and full activity was regained. The three Asp-549 mutant proteins were made in normal amounts and were readily detected both by immunoblotting analysis and ELISA. The ELISA data were used to calculate the specific activities of the lysates.

The specific activities of D549N and D549E increased ~10-fold on purification (Table I). We do not know the reason for this significant difference; however, a substantial portion of each of these two mutant proteins was lost during purification. It is possible that this portion was incorrectly folded and thus enzymatically inactive, but still was measured in the ELISA, lowering the apparent specific activity before purification. The dramatic reduction in activity observed upon mutating Asp-549 was extraordinary within cPLA$_2$, as previous to identifying the subtilisin motif, we had mutated 17 other aspartic and glutamic acid residues (E214A, D242A, E249A, E277A, D337A, D366A, multiple mutant D433A/D436A/D438A/D439A, D519A, D522A, D529A, E530A, D569A, D633A, and E658A) with no significant reduction in activity.

Histidine—We attempted to identify an essential histidine residue. Eleven of the 19 histidine residues of human cPLA$_2$ are conserved in mouse, rat, chicken, and zebra fish, and these were thus considered candidates. When each of these histidines was changed in separate mutants to alanine, significant activity always remained, suggesting that there is no essential histidine or that catalysis involves a histidine that is not conserved among species. Further mutagenesis of the remaining eight histidines proved that there is no histidine that is essential to catalysis in human cPLA$_2$ (Fig. 2). Altogether, 17 mutant forms were prepared and analyzed, one with three His-to-Ala substitutions (H9A, H14A, and H18A) and the rest with one each. All of these mutants showed >10% of wild-type activity, with some higher than wild-type activity. Adjacent to His-694 is Asp-695, reminiscent of His-Asp found in the catalytic center of the secreted PLA$_2$ enzymes. For this reason, we made H694A (designated *16* in Fig. 2) as a double mutant also containing the mutation D695A. This double mutant showed full wild-type activity.

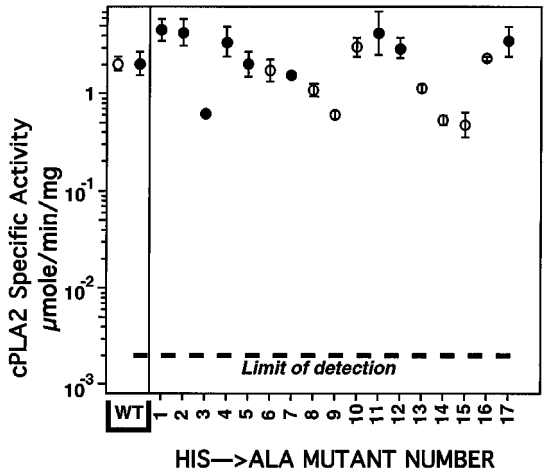

FIG. 2. **Relative enzyme activity of His-to-Ala substitution mutants of cPLA$_2$.** Lysates from 17 mutants were prepared (see "Materials and Methods") and tested for activity and ELISA as described under "Materials and Methods." Specific activities are expressed as μmol/min/mg of cPLA$_2$. The results are shown on a log scale with the minimum detectable activity level (representing 100 dpm over a typical background of 200–300 dpm) shown at the bottom. Each measurement was made with at least three independent COS cell transfections or at least three independent infections of one or more recombinant baculoviral isolates, and the standard errors of the mean are indicated. The mutants are numbered as follows: *1*, H9A/H14A/H18A; *2*, H62A; *3*, H164A; *4*, H182A; *5*, H240A; *6*, H261A; *7*, H305A; *8*, H333A; *9*, H387A; *10*, H428A; *11*, H442A; *12*, H466A; *13*, H490A; *14*, H546A; *15*, H639A; *16*, H694A/D695A; *17*, H698A. Since we have seen for wild-type cPLA$_2$ (*WT*) in many assays ~2-fold higher specific activity from COS-1 cell extracts than from insect cell extracts, all data from COS-1 cells (●) were reduced by 2-fold for comparison with the data from insect cells (○).

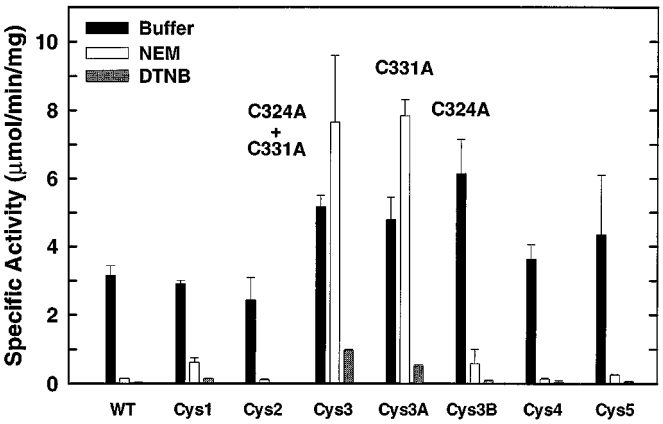

FIG. 3. **Sensitivity of Cys-to-Ala mutants to thiol-modifying agents.** The mutants are designated as follows: *Cys1*, C139A/C141A/C151A; *Cys2*, C220A; *Cys3*, C324A/C331A; *Cys4*, C620A/C634A; *Cys5*, C726A. cPLA$_2$ (10 μg/ml) in 50 mM Hepes buffer (pH 7.0), 150 mM NaCl, and 0.1% Triton X-100 was incubated at room temperature in the presence of 5 mM NEM, 1 mM DTNB, or buffer alone. After 90 min, an aliquot of this enzyme solution was diluted, and the remaining enzyme activity was determined in the phosphatidylcholine/diacylglycerol assay.

Cysteine—cPLA$_2$ is inactivated by NEM and other thiol reagents, suggesting that a cysteine may play a role in catalysis. For the nine cysteine residues of cPLA$_2$, five mutants were made (up to three Cys residues mutated in each one), and all were active. In crude lysates, all but one, the double mutant C324A/C331A, were still sensitive to NEM (data not shown). We prepared the two separate mutants C324A and C331A, purified all seven mutants, and tested their sensitivity to NEM and to DTNB (Fig. 3). Fig. 3 shows several interesting observations. (i) Only the two mutants with Cys-331 altered showed

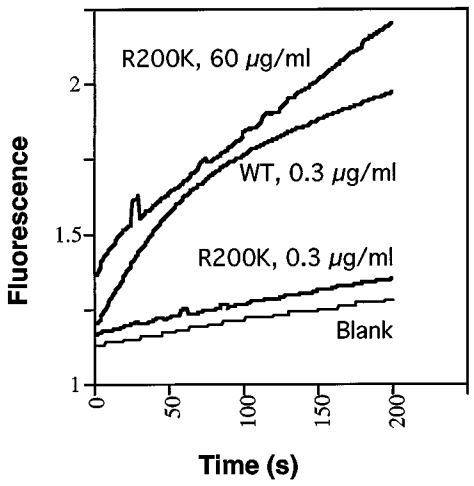

FIG. 4. **Activities of wild-type cPLA$_2$ and the R200K mutant with the 7-HCA substrate.** Hydrolysis was carried out in OSPC vesicles by purified cPLA$_2$ (wild-type (*WT*) and R200K). Each assay was in a final volume of 2 ml containing 100 μM sonicated 7-HCA/OSPC vesicles, 50 mM Tris (pH 8.0), 0.1 mM EDTA, and 1.1 mM $CaCl_2$. The concentration of 7-HCA was 10 mol %. The assay was initiated by addition of wild-type or R200K cPLA$_2$ in the amounts indicated on the *curves*. The *ordinate* is in arbitrary units of fluorescence.

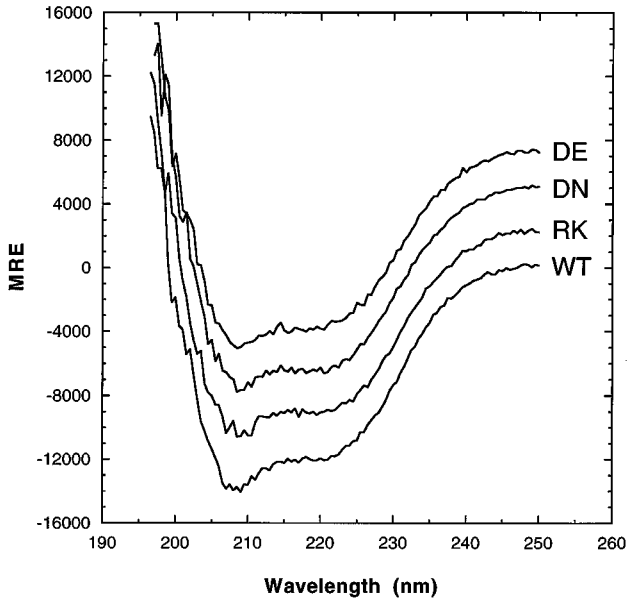

FIG. 5. **CD spectra of wild-type cPLA$_2$ (*WT*) and mutants R200K (*RK*), D549N (*DN*), and D549E (*DE*).** The spectra are offset to allow comparison. *MRE*, mean residue ellipticity.

any appreciable resistance to NEM; (ii) several of the mutants had higher initial specific activity when compared with wild-type cPLA$_2$, the highest being those with Cys-324 and Cys-331 altered; and (iii) while the DTNB treatment appreciably inactivated all the mutants, those with Cys-331 altered were significantly more resistant than the others. These results are consistent with Cys-331 having no critical catalytic role, but being situated near the catalytic center, so that when modified, it may interfere with catalysis.

Arginine—The alignment of the linear sequence of cPLA$_2$ with that of phospholipase B was a major aid in determining that Ser-228 (12) and Asp-549 (see above) were likely candidates for catalytic elements. Thus, we mutated seven additional non-aliphatic amino acids that aligned with phospholipase B (T222A, Y223A, Y238A, multiple mutant K281A/K282A/K283A, and N555A), but we found that they did not lose significant activity (data not shown). However, one that showed a striking change was Arg-200 (see Table I). When Arg-200 was substituted with alanine or histidine, there was no detectable activity, indicating that the mutants are inactivated by at least 1000-fold. Lysine was able to substitute weakly for Arg-200, with the R200K mutant having ~0.5% of wild-type activity. All three mutants (R200A, R200H, and R200K) were reverted to Arg-200 and found to regain total activity.

One possible way that arginine may contribute to catalysis is by complexing with the phosphoryl group of the phospholipid substrate, as observed in other enzymes (27–29). To test this concept, we employed a substrate devoid of phosphoryl groups, the arachidonic acid ester of 7-hydroxycoumarin (7-HCA). 7-HCA was incorporated and hydrolyzed in a mixed vesicle with the relatively inert (to cPLA$_2$) lipid OSPC as demonstrated by Huang *et al.* (5). If the function of Arg-200 were to associate with the phosphoryl groups of the substrate, one would expect that, relative to wild-type cPLA$_2$, the R200K mutant would lose considerably less activity with 7-HCA than the 200-fold observed with the phosphatidylcholine substrate. Instead, we found that R200K had still only ~0.5% of wild-type activity in this 7-HCA assay (Fig. 4). This finding suggests that Arg-200 does not simply associate with the phosphoryl group of the phospholipid molecule being hydrolyzed.

Global Conformation of Mutants—The three mutants D549N, D549E, and R200K, which had measurable catalytic activity, were purified and subjected to circular dichroism analysis. As shown in Fig. 5, the spectra appeared identical to that of wild-type cPLA$_2$, demonstrating that the significant (200–2000-fold) losses of enzyme activity are not the result of a global defect in folding of these mutants.

Evolutionary Conservation—The fact that Asp-549 and Arg-200 are among clusters of conserved residues in the alignment of cPLA$_2$ with several distantly related phospholipase B enzymes (Fig. 1, *A* and *B*) is consistent with their importance in the function of both enzymes. These two amino acids (as well as Ser-228) (12) are also conserved within the known cPLA$_2$ enzymes, lying, in fact, within regions of near total conservation among the six cPLA$_2$ sequences known (Fig. 6).

DISCUSSION

Our earlier work (12) suggested that the importance of Ser-228 for cPLA$_2$ may lie in its nucleophilic attack on the substrate, as it does in many other enzymes. More directed chemical analysis has recently been reported (13) that makes this conclusion more likely.

Lack of Histidine Requirement for Catalysis

Our data suggest that the catalytic center of cPLA$_2$ is unusual. While Ser-228 (12, 13) and Asp-549 (this work) may resemble elements of a catalytic triad, judging from their dramatic effects on enzyme activity and the amino acid sequence motifs in which they lie, there is no essential histidine residue. In light of the histidine-containing catalytic centers of the 14-kDa PLA$_2$ enzymes and of lipases for which there are three-dimensional structural data (16, 18–20), we took thorough precautions to ensure that no error had been made on the point that every His-to-Ala mutation retained significant catalytic activity and was mutated as intended. Whereas the altered sequences of all mutated DNA sequences were directly verified in their DNA vectors before recombination into the baculovirus vector, the histidine mutations that were produced in baculovirus were each verified as well in the baculovirus recombinants by analysis of polymerase chain reaction-amplified segments from the viral stocks. Data on histidine-containing catalytic triads suggest that the functional histidine is suffi-

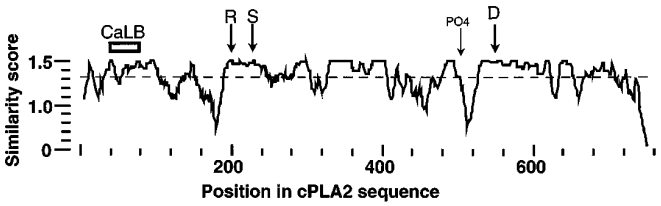

FIG. 6. **Analysis of relative similarity of six cPLA$_2$ sequences using the Genetics Computer Group program PLOTSIMILARITY (56).** The sequences compared are the translation products of DNA files with the following GenBank™ accession numbers: human (M68874), mouse (M72394), rat (U38376), guinea pig (D. L. White and J. D. Sharp, unpublished data), chicken (U10329), and zebra fish (U10330). A similarity score of 1.5 indicates perfect conservation within a local region. The locations of the catalytically essential Arg-200, Ser-228, and Asp-549 are shown by the letters *R*, *S*, and *D*, respectively, above the plot. The site of the catalytically accelerating Ser-505 is shown by *PO4* to indicate that this serine is phosphorylated when the enzyme is activated (57). The CaLB region (10), which functions for translocation to membranes in response to micromolar calcium, is indicated. The *dashed line* shows the average similarity across the entire alignment.

ciently critical for catalysis that normally no detectable activity remains when the histidine is mutated (14, 30–32). In one case in which the assay was extremely sensitive (33), mutation of the histidine was shown to reduce the activity by >5 orders of magnitude. In our case, the most severe reductions of activity among the 17 His-to-Ala mutations were <10-fold (Fig. 2).

Aspartate Role

It is remarkable that evolutionary convergence or conservation gave rise to the subtilisin aspartic acid consensus motif (26) in cPLA$_2$ and phospholipase B (Fig. 1), while no matching regions of cPLA$_2$ were found for the other subtilisin motifs (26) for the catalytic histidine or serine. Asp-549 clearly has the properties of a catalytic residue, leading to enzyme inactivation of >1000-fold when changed to alanine and of ~300- or 2000-fold when changed to asparagine or glutamic acid, respectively. It is especially interesting to compare our results with those for Asp-99 in 14-kDa pancreatic PLA$_2$ (34). The amidation of aspartate by mutation (Asp to Asn) caused an ~200-fold loss for bovine pancreatic PLA$_2$. X-ray diffraction of the D99N mutant proved that the loss of activity was explained by an alteration of the hydrogen bond scheme in which the catalytic water nucleophile was ejected (35). In cPLA$_2$, we can only speculate what change results from D549N; however, the example of pancreatic PLA$_2$ suggests some significant functional change within the catalytic center. Given the absence of an essential histidine to serve as a general base, we should entertain the possibility that Asp-549 plays this role, as, for example, does glutamate in β-lactamases (see below).

Function of Cysteines

The observation of cPLA$_2$ sensitivity to NEM led us earlier to examine the catalytic activity of the Cys-to-Ala mutants, only to find that none of the nine cysteines is essential for activity (12). Now we have shown that Cys-331 alone has the majority of the NEM sensitivity since all the other Cys-to-Ala mutants retained their NEM sensitivity, while C331A was resistant (Fig. 3). We conclude that Cys-331 may be situated near the catalytic center or at least interacts in some important way with it. Similar results have been obtained for cysteines in glutathione transferase (36) and ribonuclease H (37). The sensitivity to another thiol-modifying agent, DTNB, was also measured in these mutants (Fig. 3). While the DTNB treatment that was used inactivated all the mutants to a significant degree, there was a greater degree of resistance in C331A than was observed in the other mutants. It is notable that the specific activities of the purified untreated C331A and C324A mutants were significantly elevated over that of wild-type cPLA$_2$ (Fig. 3). The presence of these sulfhydryl groups in the native enzyme may lead to the reduction of activity in some manner. There was also a significant increase in activity upon NEM treatment of C331A and C324A/C331A, suggesting that modification by NEM of another residue (not Cys-324) may relieve partial inhibition, stabilize the enzyme, or actually activate it.

In an earlier study of this same enzyme by Li *et al.* (38), it was found that Cys-331 reacted with iodoacetamide, but that Cys-324 was the main reactant and that the enzyme was or was not inactivated by this modification depending on the conditions. Furthermore, in contrast to our results, these authors concluded that "inactivation by iodoacetamide is a consequence of labeling primarily Cys-324." Prior to submission of this manuscript, a more recent report (39) from this same group appeared in which mutants at Cys-331 and Cys-324 were analyzed in cell supernatants. These authors concluded, consistent with our results with purified enzymes, that neither cysteine is required for activity and that Cys-331 is the principal site for inhibition by thiol reagents.

Arginine Role?

Twenty-four other mutants that were prepared showed no inactivation of cPLA$_2$ below 10% of wild-type activity, but Arg-200 was quite sensitive to mutation. Any residual activity in R200A and R200H was not detectable (<1/2000 that of wild-type enzyme), and the activity of R200K was ~1/200 of wild-type cPLA$_2$. Thus, Arg-200 has a critical function for cPLA$_2$, possibly in the catalytic center. We considered the following hypothetical functions for Arg-200.

(i) Phosphoryl Group Contact—Arg-200 could be in the catalytic site and associate with the phosphoryl group of the substrate phospholipid, possibly stabilizing the transition state for formation of the acyl-enzyme. Arginine is known to interact with phosphate groups in several phosphohydrolases (*e.g.* alkaline phosphatase (27) and glycogen phosphorylase (28)) or the G-protein $G_{i\alpha1}$ (29), and Arg-53 of pancreatic PLA$_2$ (40) plays an important role in catalysis, providing specificity in interaction with the phospholipid head group. By this model, we would expect that mutation of Arg-200 to lysine (the R200K mutant) would show, as it does, a significant drop in activity. But we would also expect that hydrolysis of the coumarin ester would be less sensitive to this mutation, and that was not the case (Fig. 4).

(ii) Transition State Stabilization—Arg-200 could be in the catalytic site and play a role in stabilizing the oxyanion for acyl-enzyme hydrolysis. The oxyanion hole of hydrolytic enzymes has quite a varied composition. For example, in pancreatic PLA$_2$, the oxyanion hole is formed by Ca^{2+} and the backbone amide of a glycine residue (41). In carboxypeptidase A, it was traditionally established that a Zn^{2+} ion plays this role, but recent data implicate Arg-127 of that enzyme instead (42). Since the oxyanion is the same for arachidonoylphospholipid as for 7-HCA, our result is consistent with this role for Arg-200, showing that R200K has lost ~200-fold activity for both substrates.

(iii) Catalytic Triad—Arg-200 could be in the catalytic site and play a catalytic role, like histidine in conventional catalytic triads, in polarizing Ser-228 for nucleophilic attack on the fatty acyl group. While one might be attracted to this choice simply because of the vacuum left by the absence of an essential histidine, it may be difficult to envision arginine functioning in a hydrogen-bonding network because of its extremely high pK_a (12.5). Recent advances in understanding the energetics of

"low-barrier hydrogen bonds" formed within hydrogen-bonding networks of catalytic triads have revealed that a great deal of energy can be derived from the close matching of pK_a values between His and Asp, modified within the enzyme to become nearly identical in specific cases (43–45). While one may conceive of such a low-barrier hydrogen bond between the nucleophilic Ser-228 and arginine, made possible by their close pK_a values (13.5 for serine and 12.5 for arginine), more direct structural or kinetic evidence will be required to establish that relationship. Still, just such a proton extraction from a nucleophilic cysteine was postulated for arginine in the case of the acyl-acylthioesterases (30), in which substitution of the putative catalytic histidine with arginine resulted in a drastic shift of pH optimum with significant retention of catalytic activity, particularly at pH values approaching 12. This is interesting as the pH optimum of cPLA$_2$ is somewhat alkaline, being centered around 9–10 (46), and significant shifts of pK_a values are known within the environment of enzyme structures (47).

We are not aware of any hydrolytic enzyme with a catalytic triad in which arginine substitutes for histidine, other than the artificial substitution in the thioesterase just mentioned. Lysine has been implicated in a related role in signal peptidases (48, 49), in class A β-lactamases (50), and in the autoproteolytic lexA repressor (51, 52), forming a catalytic dyad with a serine nucleophile. In one exceptional case based on the x-ray crystal structure of penicillin acylase (53), a lone serine residue has been postulated to function as the nucleophile without any assistance other than possibly its own (terminal, free) amino group. For the β-lactamases, the role of lysine has been questioned, and recent evidence (54) suggests that, in this case, glutamate acts directly as the general base to extract the proton from the serine nucleophile.

(iv) CaLB Assistance—Arg-200 could interact with the CaLB (C2) domain to assist in binding the enzyme to the lipid interface. This role must formally be entertained since it has been shown that activation by calcium is an important, and biologically possibly essential, part of cPLA$_2$'s function. Although from our data it cannot be ruled out that Arg-200 functions in this way, it has been shown conclusively (10) that the region from amino acids 1 to 134 is sufficient to respond to calcium and translocate to the membrane. Arg-200 is not within this functional region of the sequence.

(v) Interface with Phospholipid Surface—Arg-200 could be involved in the enzyme's association with phosphoryl groups of phospholipids in the interface (55). This role would be distinct from phosphoryl group contact and CaLB assistance, referring to the possible direct interaction of Arg-200 with the aggregated lipid surface. In the experiment with the 7-HCA ester, the presentation of the substrate includes the phospholipid OSPC, which is rather inert to cPLA$_2$, but which still has the phosphoryl groups that might interact with Arg-200. Therefore, we must conclude that this option is left open.

(vi) Other Roles—There could be some other instrumental role that Arg-200 might play in cPLA$_2$ function. Although the CD spectra make it seem less likely that the R200K mutant is inactive because of a global defect in protein folding, this technique is not sensitive to subtle changes that do not affect the overall content of α- and β-structure within the protein. Thus, for example, a critical salt bridge may be disrupted by changing Arg-200, or some contact with the Arg-200 side chain may be critical for formation of the structure around the catalytic center. Our data do not give much guidance for such possibilities; however, the observation that lysine can replace Arg-200 with some retention of activity while histidine and alanine cannot suggests the importance of positive charge at that position.

CONCLUSION

Whatever mechanism may eventually be established for cPLA$_2$, it appears that this catalytic center has some novel elements, and although it catalyzes the identical catalytic cleavage on a heavily overlapping set of phospholipid substrates as do the small 14-kDa secreted PLA$_2$ enzymes, its mechanism appears to have some significant differences. In the small PLA$_2$ enzymes, the critical catalytic elements are histidine, aspartic acid, and calcium, while in cPLA$_2$, the known essential elements are now serine, aspartic acid, and arginine, with calcium being nonessential for catalysis (4). A number of carboxylesterases including lipases have been shown by structural and mutagenic evidence to have more conventional catalytic triads involving histidine. Examples are human pancreatic lipase (16), two fungal triacylglycerol lipases (18, 19), acetylcholinesterase (20), and platelet-activating factor acetylhydrolase, which also has PLA$_2$ activity (14). This clearly cannot be the case for cPLA$_2$.

In summary, we have eliminated the possibility that histidine or cysteine plays an essential role in phospholipid hydrolysis by cPLA$_2$, and we have found that Asp-549 and Arg-200, and previously Ser-228 (12), are essential for the reaction. Based on the conservation of these three critical residues in phospholipase B, we further speculate that the catalytic elements of these enzymes may be the same. We conclude that the cPLA$_2$ catalytic center has two novel features. (i) The catalytically essential Asp-549 is found within a perfect copy of the catalytic aspartic acid motif of the subtilisin family of proteases, and (ii) there is no essential histidine in cPLA$_2$.

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Identification of Essential Residues for the Catalytic Function of 85-kDa Cytosolic Phospholipase A $_2$: PROBING THE ROLE OF HISTIDINE, ASPARTIC ACID, CYSTEINE, AND ARGININE

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