Articles

The Crystal Structure of the H48Q Active Site Mutant of Human Group IIA Secreted Phospholipase A_2 at 1.5 Å Resolution Provides an Insight into the Catalytic Mechanism[†,‡]

Suzanne H. Edwards, Darren Thompson, Sharon F. Baker, Stephen P. Wood, and David C. Wilton*

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K.

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ABSTRACT: The human group IIA secreted PLA_2 is a 14 kDa calcium-dependent extracellular enzyme that has been characterized as an acute phase protein with important antimicrobial activity and has been implicated in signal transduction. The selective binding of this enzyme to the phospholipid substrate interface plays a crucial role in its physiological function. To study interfacial binding in the absence of catalysis, one strategy is to produce structurally intact but catalytically inactive mutants. The active site mutants H48Q, H48N, and H48A had been prepared for the secreted PLA_2s from bovine pancreas and bee venom and retained minimal catalytic activity while the H48Q mutant showed the maximum structural integrity. Preparation of the mutant H48Q of the human group IIA enzyme unexpectedly produced an enzyme that retained significant (2−4%) catalytic activity that was contrary to expectations in view of the accepted catalytic mechanism. In this paper it is established that the high residual activity of the H48Q mutant is genuine, not due to contamination, and can be seen under a variety of assay conditions including assays in the presence of Co^{2+} and Ni^{2+} in place of Ca^{2+}. The crystallization of the H48Q mutant, yielding diffraction data to a resolution of 1.5 Å, allowed a comparison with the corresponding recombinant wild-type enzyme (N1A) that was also crystallized. This comparison revealed that all of the important features of the catalytic machinery were in place and the two structures were virtually superimposable. In particular, the catalytic calcium ion occupied an identical position in the active site of the two proteins, and the catalytic water molecule (w6) was clearly resolved in the H48Q mutant. We propose that a variation of the calcium-coordinated oxyanion ("two water") mechanism involving hydrogen bonding rather than the anticipated full proton transfer to the histidine will best explain the ability of an active site glutamine to allow significant catalytic activity.

Secreted phospholipases A_2 ($sPLA_2$)[1] are a ubiquitous family of small (14 kDa), disulfide-containing calcium-dependent enzymes that catalyze the hydrolysis of the *sn*-2 ester bond of phospholipids, liberating lysophospholipid and free fatty acid products. Apart from being the products of phospholipid digestion, fatty acids and lysophospholipids may be transformed into inflammatory lipid mediators, thereby implicating $sPLA_2$s in the pathogenesis of many inflammatory disease states. In fact, $sPLA_2$s are found in a variety of extracellular locations and are involved in a range of physiological processes including phospholipid digestion, signal transduction, and host defense (*1*).

Human group IIA $sPLA_2$ was first isolated from the synovial fluid of patients suffering from rheumatoid arthritis (*2*) and has since been found in a variety of cells, including platelets, macrophages, spleen, smooth muscle, and placenta (*1*). It is believed to be an acute phase protein (*3*), with a role in the removal of damaged/apoptotic cells (*4*, *5*) and in host defense against bacterial infection (reviewed in ref *6*). The highly cationic nature of the enzyme together with its preference for phospholipids present in anionic interfaces and its negligible activity with zwitterionic interfaces as seen with the mammalian cell membrane and lipoproteins is well suited to its antibacterial role in the body (*7*). This specificity for bacterial membranes is due to the interfacial binding characteristics of the enzyme and provides a dramatic

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[‡] The atomic coordinates and structure factors (code 1N28 for H48Q and 1N29 for the wild-type (N1A) human group IIA enzymes) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

* To whom correspondence should be addressed. Phone: +44 (0)-2380 594308. Fax: +44 (0)2380 594459. E-mail: D.C.Wilton@soton.ac.uk.

[1] Abbreviations: DAUDA, 11-(dansylamino)undecanoic acid; FABP, fatty acid-binding protein; $sPLA_2$, secreted phospholipase A_2; pyrene-PG, 1-palmitoyl-2-(10-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol; DOPG, dioleoylphosphatidylglycerol; HBSS, Hank's balanced salt solution; dansyl-DPPE, *N*-dansyl-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; ESI-MS, electrospray ionization mass spectrometry; SUV, small unilamellar vesicle.

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example of the importance of interfacial binding in physiological regulation.

The proposed mechanism of catalysis of $sPLA_2$s involves the His-48/Asp-99 pair found at the active site. The basic mechanism of this catalytic dyad involves the activation of a water molecule by histidine, thus facilitating nucleophilic attack on the ester carbonyl at the *sn*-2 position, producing an oxyanion intermediate. Subsequent collapse of this oxyanion intermediate gives the products, fatty acid and lysophospholipid. The active site Ca^{2+} is coordinated to Asp-49 as well as other active site residues and is involved both in substrate binding and in stabilization of the oxyanion transition state during catalysis (*8*). One or two water molecules are seen at the active site of crystal structures, and the precise implications of these water molecules in terms of mechanism have recently been discussed (*9*).

In the case of the pancreatic enzymes, active site mutants of both the bovine and porcine enzymes have been evaluated. In particular, the histidine mutants have been prepared and have been found to have very low activity. The H48Q and H48N mutants of the bovine enzyme have less than 0.00014% and 0.006% of the wild-type activity, respectively (*10*), while the porcine enzyme H48Q and H48N mutants have 0.0016% and 0.00068% (*11*). In both cases activity was measured with diC8PC micelles using proton release (pH stat) assays. The very low activity seen with these histidine mutants coupled with the fact that in the case of the H48Q mutants the proteins were structurally intact (*10*, *11*) should allow such mutants to be used in interfacial competition assays (*12*). In these assays the inactive protein competes with an active enzyme for the phospholipid surface. In addition, such mutants should permit the identification of physiological roles for the IIA enzyme that do not require cell phospholipid hydrolysis.

In this paper we describe the preparation of the H48Q and H48N mutants of the human group IIA $sPLA_2$ and demonstrate the considerable activity of the H48Q enzyme (*13*) and significant activity of the H48N enzyme. The crystal structure of the H48Q at 1.5 Å reveals that the active site structure is maintained intact in the mutant and suggests a mechanism in which it is not essential to involve a base (His-48) to formally deprotonate a water molecule in order to facilitate catalysis.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Customized oligonucleotides were designed on the basis of the gene sequence of human $sPLA_2$ and produced by Oswel (Southampton, U.K.). Forward (5′ end, A): AAAA GAA TTC CAT ATG GCC CTG GTA AAC TTC CAC. Reverse (3′ end, B): AAAA AAG CTT ACT ATT AGC AAC GC. H48A (A*): GTA GCA GCA GTC **AGC** CGT AAC GCA GCA. H48A (B*): TGC TGC GTT ACC **GCT** GAC TGC TGC TAC. H48Q: TGC TGC GTT ACC **CAG** GAC TGC TGC TAC. H48N: TGC TGC GTT ACC **AAC** GAC TGC TGC TAC. (Mutations are shown underlined in bold.)

Site-directed mutagenesis was effected using a two-stage PCR reaction (*14*) (H48A) or the Kunkel method (*15*) (H48Q, H48N). The two-stage PCR mutagenesis introduces the required mutation into the gene by priming the native gene with oligonucleotides containing the mutation. Two separate reactions are used to produce DNA fragments containing the mutation, which can then be extended into full-length mutant DNA by further PCR. Reaction 1 contained 200 nmol of PLA_2 gene template, 100 nmol each of primers A and A*, 16 nmol of dNTPs, and H_2O to a total volume of 50 μL. In reaction 2 primers A and A* were replaced by primers B and B*. After 1 min at 94 °C, 2.5 units of *pfu* DNA polymerase was added, and 25 cycles of PCR were performed under the following conditions: 94 °C (1 min), 66 °C (1 min), and 72 °C (1 min). Equal molar quantities of reactions 1 and 2 were then combined, and the PCR was repeated as above using 5′ and 3′ primers (A and B) to produce a continuous length of DNA containing the mutation. The PCR reaction was then purified using a QIAquick PCR purification kit, and the mutant DNA was cloned into the pET11a vector for expression.

Overexpression and Purification of Human Group IIA $sPLA_2$. The human group IIA $sPLA_2$ was overexpressed and purified as reported previously (*16*). This enzyme is expressed in *Escherichia coli* from a synthetic gene as an N1A mutant. This mutant has properties essentially identical to those of the wild-type enzyme while the N-terminal alanine ensures that the *E. coli* aminopeptidase removes the initiator methionine (*16*, *17*). The H48Q, H48N, and H48A mutants are therefore all double mutants (N1A, H48Q; N1A, H48N; N1A, H48A).

Measurement of PLA_2 Activity Using a Continuous Fluorescence Displacement Assay. The fluorescence assay was performed as described previously (*18*, *19*). Each assay contained 0.1 M Tris·HCl, pH 8.0, 0.1 M NaCl, 2.5 mM $CaCl_2$, 1 μM DAUDA, 50 μg/mL phospholipid, and ~12 μg/mL FABP. The reaction was started by the addition of PLA_2. Assays were calibrated by the addition of a known amount of fatty acid, and all assays were performed in triplicate.

Measurement of PLA_2 Activity Using Pyrene-PG. The substrate, pyrene-PG, was presented as SUVs (*20*). A typical assay contained 1.5 mL of buffer T (50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1 mM EGTA), 2 μM pyrene-PG, 15 μL of 10% fatty acid-free BSA, and the required amount of $sPLA_2$. The reaction was started by the addition of 15 μL of 1 M $CaCl_2$. The maximum fluorescent signal was obtained in a separate experiment by addition of 5 μg of *Naja naja* snake venom $sPLA_2$. The assay was calibrated by titrating10-pyrenyldecanoic acid into the system.

Measurement of $sPLA_2$ Activity Using Suspensions of Micrococcus luteus. Single bacterial colonies were grown overnight in 10 mL of LB broth at 37 °C with shaking. The overnight culture was diluted 10-fold and grown to OD_{600} ~0.45. The bacteria were centrifuged at 2500*g* for 15 min and resuspended in ~2.5 mL of HBSS so that the OD_{600} of a 20-fold dilution in HBSS was ~0.11. This absorbance equates to an approximate bacterial concentration of 2×10^7 cells/mL or ~1 nmol of phospholipid/mL of assay (*21*). The bacteria were kept on ice until required. The assay was carried out at 37 °C using a Hitachi F-2000 fluorescence spectrophotometer. A typical assay contained 1 mL of HBSS, 1 μM DAUDA, ~12 μg/mL FABP, 2.5 mM $CaCl_2$, and 2×10^7 cells/mL. The reaction was started by addition of the required amount of $sPLA_2$.

Measurement of $sPLA_2$ Activity Using a pH Stat. Specific activities were calculated by pH stat titration with a

Radiometer PHM290 pH stat controller and an ABU901 autoburet at room temperature. Substrate vesicles were prepared by drying a mixture of 3 mM diC8PC/3 mM taurodeoxycholate under N_2 and resuspending the lipid film in assay buffer (0.5 mM Tris·HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$). Aliquots of 3 mL were used to measure activity, and measurements were started after the baseline drift had stabilized (~1−4 min) by addition of the appropriate amount of enzyme. All assays were performed in triplicate.

Binding Studies Using Dansyl-DPPE. The fluorescent probe dansyl-DPPE was incorporated into vesicles at 4 mol % by drying a mixture of 15 μM DOPG and 0.2 μM dansyl-DPPE under a stream of nitrogen. Phospholipid was resuspended in methanol, and vesicles were formed by rapid injection into reaction medium (0.1 M Tris·HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA) (*16*). Fluorescence measurements were performed in triplicate after each addition of enzyme using a Hitachi F-2000 fluorescence spectrophotometer, with excitation at 345 nm and emission measured between 450 and 600 nm.

CD Spectra. The CD spectra of enzymes were measured using a Jasco J-720 spectropolarimeter. For comparative secondary structure CD, 300 μL of a 0.2 mg/mL solution of protein in 10 mM phosphate buffer, pH 7.4, was measured between 190 and 250 nm. Spectra were averaged from seven accumulations.

Crystallography. Crystals of N1A were grown at 4 °C from droplets containing ~10 mg/mL protein, 5 mM Ca^{2+}, 0.1 M Tris·HCl (pH 7.5), 5.5 M NaCl, and 0.5 mM octyl β-glucoside. A single crystal, in a 50:50 solution of well buffer and PEG 550 MME, was used for data collection, which proceeded through 60°, with an oscillation angle of 1°. Diffraction data were collected at 100 K on a MAR image plate, using monochromatic Cu Kα radiation (λ = 1.5418 Å) from a rotating anode generator for the recombinant wild type (N1A), or were collected at a Synchrotron source (Grenoble) (H48Q). The N1A crystal belonged to space group $P6_122$, as determined by the symmetry and systematic absences in the diffraction pattern. The unit cell parameters were a = 74.79 Å, b = 74.79 Å, and c = 88.891 Å, and there was one molecule in the asymmetric unit. The data set contains 31180 reflections (4693 unique), with an R_{merge} of 0.066 and a completeness of 99.5% at 2.6 Å. Initial phases were calculated from a molecular replacement solution obtained with the wild-type PLA_2 [PDB entry 1POD (*22*)] as the search model. Rigid body refinement was done in CNS (*23*). The program QUANTA 96 (Molecular Simulations, San Diego, CA) was used for visualization purposes. The initial R-factor was 0.33 (R_{free} = 0.38). Crystals of the H48Q were grown at 4 °C as the N1A (see above), except that the [NaCl] was 5.3 M and the pH 7.4. A single crystal was used for data collection (for conditions, see above), which proceeded through 180°, with an oscillation angle of 1°. The H48Q crystal belonged to space group $C2$, as determined by the symmetry and systematic absences in the diffraction pattern. The unit cell parameters were a = 119.58 Å, b = 34.42 Å, and c = 73.91 Å, with a unique β angle of 126.6°, and there were two molecules in the asymmetric unit. The data set contains 161166 reflections (37032 unique), with an R_{merge} of 0.089 and a completeness of 83.6% at 1.5 Å. The 2.2 Å resolution crystal structure of wild-type IIA $sPLA_2$ was used as a search model as above. The initial R-factor was 0.41 (R_{free} = 0.42). For both structures Xtalview (*24*) or Molscript (*25*) was used with Raster3d (*26*) to generate pictures.

RESULTS

Production of H48A, H48N, and H48Q Group IIA $sPLA_2$s. Three active site mutants of human group IIA $sPLA_2$ were produced using site-directed mutagenesis in which the catalytic residue His-48 was substituted with Ala (H48A), Asn (H48N), or Gln(H48Q). The resulting mutants were sequenced to confirm the presence of the desired mutation, and mutant protein was then prepared and purified as detailed in Materials and Methods. The H48N and H48Q mutants expressed normally and yielded approximately 7 mg of pure protein/L of broth, which is in good agreement with the yield of the wild-type enzyme. The amino acid composition of these mutants was established by ESI-MS (H48Q, 13852 ± 1.0, predicted 13851; H48N, 13838 ± 1.0, predicted 13837) while CD spectra (data not shown) confirmed the overall structural integrity of the mutants. No folded H48A protein could be obtained, indicating that the substitution of a histidine residue with an alanine had a serious effect on the structural integrity of the enzyme. This is consistent with the previously reported H48A mutant of the bovine pancreatic (*10*) and the equivalent H34A mutant of the bee venom enzyme (*27*). As a result, no further studies were carried out on this particular mutant.

Catalytic Activity of the H48Q and H48N Mutants of Human Group IIA $sPLA_2$. Initial measurements were performed on SUVs prepared from DOPG using a fluorescent displacement assay (*18*, *19*). The rates of hydrolysis were independent of substrate concentrations over the range of 25−250 μM, and these results are consistent with the expected very high affinity of these enzymes for anionic interfaces, resulting in scooting kinetics (*9*). The results of such assays are shown in Table 1 and highlight the considerable activity of the H48Q mutant at 2.8% of the wild-type enzyme. Such a value is 1000−10000-fold greater than that seen with the corresponding H48Q mutants of the group I porcine and bovine pancreatic enzymes (*10*, *11*). Moreover, the H48N mutant also showed significant activity although it was ~10-fold lower than the H48Q (Table 1). To ensure lack of enzyme contamination, the H48Q mutant was recloned several times and re-expressed from single colonies without loss of this activity.

To ensure that the activity of the H48Q mutant was not due to an artifact of the assay procedure, several different types of assays were performed, and the results are summarized in Table 1. The Radvanyi assay (*20*) involves the use of pyrene-labeled phospholipid substrate and the measurement of released pyrene fatty acid. This pyrene fatty acid gives pyrene monomer fluorescence as a result of binding to albumin present in the assay buffer. It is a direct assay where an increase in fluorescence is measured, and this assay produced an activity for the H48Q mutant that was 2.9% of the wild-type enzyme.

A direct measurement of enzyme activity was also determined by monitoring proton release using a pH stat. In this case we found the best substrate to be diC8PC, presented as an anionic mixed micelle with taurodeoxycholate. Using

Table 1: Specific Activity of Recombinant Wild-Type (N1A) Human Group IIA $sPLA_2$ and Mutants H48Q and H48N Using a Variety of Enzyme Assays[a]

method	specific activity (nmol min^{-1} μg^{-1})			activity of H48Q as % of N1A	activity of H48N as % of N1A
	N1A	H48Q	H48N		
fluorescence displacement assay	64.35 ± 4.75	1.80 ± 0.01	0.143 ± 0.004	2.80	0.22
pH stat	235.00 ± 20.87	9.78 ± 1.57	0.78 ± 0.14	4.16	0.33
pyrene-labeled phospholipid	112.00 ± 19.95	3.20 ± 0.75		2.86	
M. luteus	1.94 ± 0.34	0.06 ± 0.003		3.09	

[a] Specific activities of the N1A, H48Q, and H48N mutants of group IIA $sPLA_2$ were measured using the methods indicated. In the case of the fluorescence displacement assay (*18*, *19*) 64 μM DOPG SUVs were used as substrate. For the pH stat assays (*10*, *11*), micelles consisting of 3 mM dioctanoyl-PC/3 mM taurodeoxycholate were employed. The pyrene-PG assay (*20*) contained 2 μM vesicles, and the *M. luteus* assay (*7*, *21*) contained ~1 μM phospholipid. All assays contained calcium (1−2.5 mM). Data shown are means ± SD; $n = 3$.

diC8PC had the added advantage of being a different (zwitterionic) substrate that was also used in studies with the pancreatic enzymes (*10*, *11*). Under these assay conditions the specific activity for the H48Q was 4.2% that of the wild-type enzyme (Table 1). For all enzyme assays no activity is seen in the absence of Ca^{2+} ions produced by including excess EGTA (data not shown).

Hydrolysis of M. luteus Cell Membranes by Wild-Type Enzyme and the H48Q Mutant. The antibacterial properties of the human group IIA $sPLA_2$ are now well established and linked to the ability of the enzyme to hydrolyze phospholipids in the cell membrane of Gram-positive bacteria (reviewed in ref *6*). The fluorescent displacement assay allows a direct measure of such hydrolysis using bacterial suspensions (*7*, *21*) and provided a unique opportunity to compare the activity of the wild-type and H48Q mutant enzymes using a physiological substrate. Under these assay conditions (Table 1) the H48Q had 3.1% of the activity of the wild-type enzyme, thus supporting the results showing high H48Q activity using artificial substrates.

Cation Specificity of the Group IIA $sPLA_2$ and the H48Q Mutant. In addition to Ca^{2+}, the pancreatic enzyme can express significant activity with Co^{2+} and Ni^{2+} (*28*), and these cations are also able to support the activity of the group IIA enzyme (*28*). The ability of the H48Q mutation of the group IIA enzyme to express considerable activity suggests that there is minimal overall perturbation of the active site. If this were the case, then the H48Q mutant would also be able to use Co^{2+} and Ni^{2+} in place of Ca^{2+}. Moreover, the expressed activity with each divalent cation should be a similar percentage of the rate seen with $Ca^{2+}$when comparing the wild-type and H48Q mutant enzymes if the role of the divalent metal cation is unperturbed with the H48Q.

To overcome the problem of residual Ca^{2+} in assay buffers, assays with DOPG vesicles were performed in the absence of added Ca^{2+} and in the presence of sufficient EGTA to remove residual Ca^{2+} so that the catalytic rate was reduced to zero. Enzyme activity was then assayed in the presence of Ca^{2+}, Co^{2+}, and Ni^{2+} up to a concentration of 200 μM cation in order to keep the Ni^{2+} concentration below 300 μM (*28*). The specific activity was determined for both the wild type and H48Q mutants, and the results (Figure 1) clearly show that the H48Q mutant was able use both Co^{2+} and Ni^{2+}. Moreover, the rates that were observed were very similar as a proportion of the Ca^{2+} rate for both the wild type and H48Q mutant. The specific activities for the wild type and H48Q mutant when assayed at 1 mM added Ca^{2+} are also shown (Figure 1) and reflect the extracellular free Ca^{2+} concentration normally experienced by the enzyme

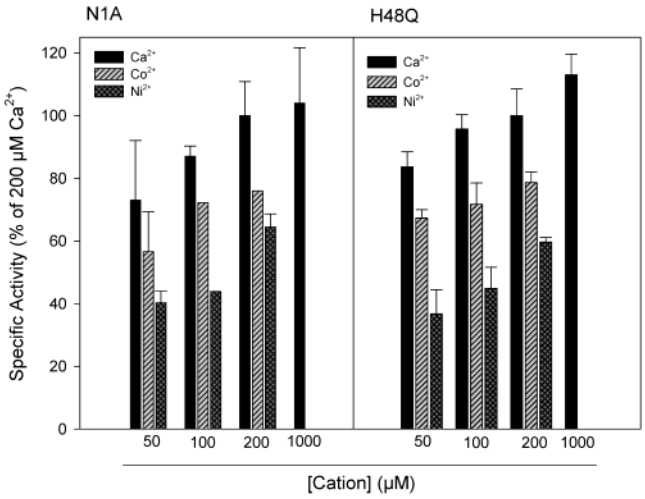

FIGURE 1: Activity of recombinant wild-type $sPLA_2$ (N1A) and the H48Q mutant assayed in the presence of Ca^{2+}, Co^{2+}, and Ni^{2+}. Specific activities were determined for the wild type and the H48Q mutant using the fluorescence displacement assay with SUVs of DOPG and the indicated amount of either Ca^{2+} (solid bars), Co^{2+} (diagonal hatched bars), or Ni^{2+} (crosshatched bars). Data shown are means ± SD; $n = 3$.

under physiological conditions. These results provide clear evidence in support of the proposal that that there is no major perturbation of cation binding at the active site in the H48Q mutant.

It is not known if Co^{2+} and Ni^{2+} are able to replace Ca^{2+} in the secondary binding site found in the group IIA $sPLA_2$ (*22* and see below). However, similar results were obtained with these metal ions when comparing the bovine and porcine pancreatic enzymes (*28*) even though a secondary calcium binding site is not seen with the bovine enzyme (*9*).

Binding of Wild-Type Enzyme and the Mutants H48Q and H48N to Anionic Vesicles. The binding of the wild type and mutant enzymes to anionic vesicles was assessed by using SUVs of DOPG containing 4 mol % dansyl-PE, in which the dansyl fluorophore is attached to the ethanolamine headgroup of the phospholipid. When incorporated into a bilayer, the dansyl group of the dansyl-PE will be solvent-exposed at the interface with the aqueous phase. When an enzyme binds to the interface, desolvation of the probe occurs with a blue shift in emission wavelength and an increase in fluorescence intensity (*16*, *29*, *30*). The result of titrating enzyme into a fixed quantity of phospholipid is shown in Figure 2, where assays were performed in the presence of EDTA to prevent phospholipid hydrolysis. It is established that Ca^{2+} is not required for the interfacial binding of these enzymes (*31*). The results clearly show that the wild-type

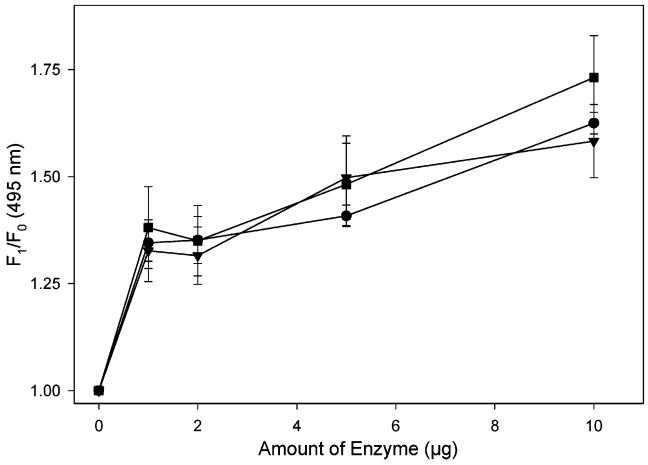

FIGURE 2: Comparison of the binding of recombinant wild type (N1A) and the H48Q and H48N forms of human group IIA $sPLA_2$ to vesicles of DOPG containing the fluorescent phospholipid probe dansyl-DPPE. The preparation of 15 μM DOPG vesicles containing 4 mol % dansyl-DPPE is described in Materials and Methods. Enzyme binding was monitored as an increase in fluorescence at 495 nm as a result of desolvation of the dansyl fluorophore. All assays were performed in the absence of added Ca^{2+} and contained 1 mM EDTA. Key: (●) N1A; (▼) H48Q; (■) H48N. Data shown are means ± SD; $n = 3$.

enzyme and the H48Q and H48N mutants show very similar and tight binding to the phospholipid vesicles.

Crystal Structure of the Recombinant Wild Type (N1A) and H48Q Mutants of Human Group IIA sPLA₂. The remarkable catalytic properties of the H48Q mutant required a detailed knowledge of the active site structure of this mutant in order to provide a structural basis for mechanistic proposals. Moreover, because the synthetic human group IIA gene has been designed to produce an N1A mutant in order to facilitate expression in *E. coli*, it was an opportune time to unambiguously confirm that this recombinant wild type (N1A mutant) was essentially identical to the natural enzyme in terms of structure.

Crystallography of the N1A and H48Q forms of group IIA $sPLA_2$ was carried out as described in Materials and Methods. A summary of the pertinent crystallography data is shown in Table 2. In particular, a resolution of 1.5 Å was achieved for the H48Q mutant and is one of the highest resolutions achieved for group IIA $sPLA_2$s to date.

The C-α backbone trace of the H48Q and the wild type (N1A) was superimposed onto that of the original wild-type enzyme (*28*), and this showed that all regions of the group IIA mutants were in exactly the same conformation as the original wild-type enzyme [RMSD of 0.769 Å (H48Q) and 0.507 Å (N1A)]. The secondary structure of H48Q superimposed onto that of the recombinant wild-type (N1A) structure demonstrates that the two structures are essentially identical (RMSD between the two structures is 0.686 Å for all α carbon atoms). The two Ca^{2+} ions found in both models (see below) can also be seen and occupy identical positions (data not shown). Some regions of the H48Q chain are slightly different from the N1A mutant, but as the resolution of the H48Q model is much higher (1.5 Å compared with 2.6 Å), these areas may actually represent the more accurate conformation. Also, as the crystals are from different space groups, slight differences may represent differences in contacts due to the crystal packing.

Table 2: Crystal Data for the Two Mutants of Human Group IIA $sPLA_2$

type of enzyme	N1A[a]	H48Q[a]
space group	$P6_122$	$C2$
cell parameters (Å)	$a = b = 74.79$, $c = 88.89$	$a = 119.58$, $b = 34.42$, $c = 73.91$
cell angles (deg)	$\alpha = \beta = 90$, $\gamma = 120$	$\alpha = \gamma = 90$, $\beta = 126.56$
resolution range (Å)	15−2.6	20−1.5
R-factor (*R*-free), initial	0.3324 (0.3755)	0.4149 (0.4209)
R-factor (*R*-free), final	0.2423 (0.3197)	0.2565 (0.2732)
no. of reflections measured	31180	161166
no. of unique reflections	4693	37032
R_{sym}	0.066	0.089
multiplicity	3.4	2.4
completeness (%)	99.5	83.6

[a] The N1A mutant refers to the recombinant wild-type enzyme expressed in *E. coli* and which in terms of kinetic properties appears to be identical to the wild-type enzyme. All further mutations of the protein are compared with N1A. Thus the H48Q mutant is in fact N1A, H48Q when compared with the wild-type enzyme.

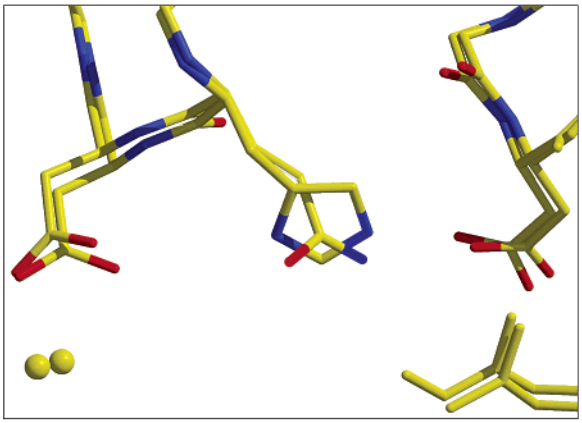

FIGURE 3: Comparison of the active site dyad and calcium binding residues of the recombinant wild-type (N1A) $sPLA_2$ and the H48Q mutant. The models show the almost identical positioning of the Asp residues at positions 49 and 99 and the His/Gln at position 48 in the N1A and H48Q structures. The calcium ions coordinated by Asp-49 are shown as yellow spheres.

Inspection of the active site of the H48Q revealed that there was no significant perturbation of the catalytic machinery. Figure 3 is a global comparison of the active sites of H48Q and recombinant wild type (N1A) showing position 48, the calcium binding Asp-49 residue, and the Asp-99 residue that completes the catalytic pair. The two structures are very similar in terms of the positioning of the residues and the bound water and calcium molecules. The fine structure of Gln-48, Asp-99, and the two associated catalytic water molecules is shown in Figure 4. Two water molecules are seen which are clearly associated with the glutamine residue, and these occupy the same space structurally as those in the wild-type (N1A) model.

It is clear from Figure 4 that the orientation of Gln-48 has the carbonyl oxygen of the amide group orientated toward the water molecule (w6) while the nitrogen is orientated toward Asp-99. This orientation is consistent with that reported for the crystal structure of the H48Q mutant of the bovine pancreatic enzyme (*32*). Such an orientation will

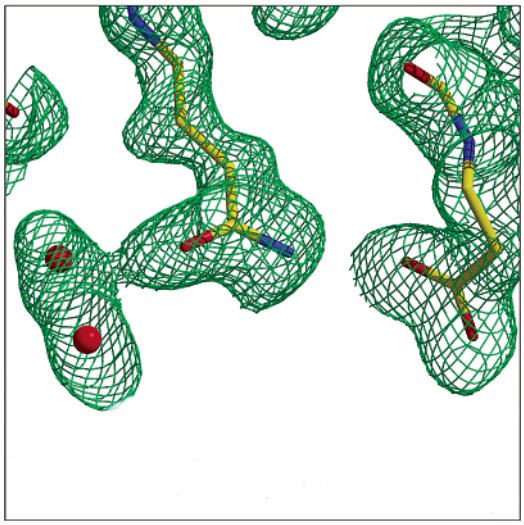

FIGURE 4: Active site dyad of H48Q $sPLA_2$. The active site dyad of Gln-48 and Asp-99 is shown for the H48Q mutant. Two water molecules, w6 and w7, are shown to be associated with the Gln-48 residue. Electron density is shown for a $2F_o - F_c$ map at the 1σ level. Red spheres represent water molecules. It is the carbonyl oxygen of the amide that hydrogen bonds to w6, the catalytic water molecule.

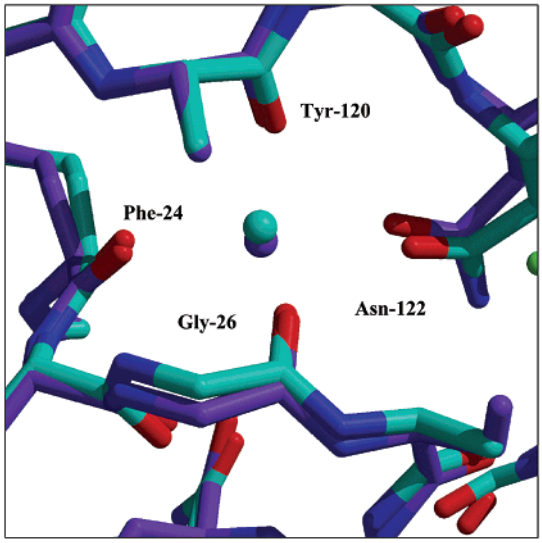

FIGURE 5: Comparison of the secondary calcium ion binding of recombinant wild-type (N1A) and H48Q $sPLA_2$s. The secondary structure of the wild type (N1A) is in purple and that of the H48Q is in blue.

allow a partial proton transfer from w6 as a hydrogen bond to the carbonyl oxygen. The resonance of the side chain amide bond of Gln-48 has implications for the mechanism (see below).

The calcium binding residues (Asp-49 and the carbonyl oxygen of residues at positions 28, 30, and 32) are in identical positions in both the H48Q and N1A structures, and density for a calcium ion could be assigned in both models (Figure 3). The His-48 residue is adjacent to the Asp-49 residue that coordinates calcium as part of the catalytic mechanism. The substitution of this histidine with a glutamine has not, therefore, impaired the calcium binding ability of the mutant. This is completely consistent with the kinetic studies where both Co^{2+} and Ni^{2+} are able to substitute for Ca^{2+} to a similar extent in facilitating hydrolysis in the wild-type and H48Q enzymes (Figure 1).

The crystal structures of the N1A and the H48Q revealed electron density for a second calcium ion in the models (Figure 5). This calcium is coordinated by Asn-122 and the carbonyl oxygen of residues 24, 26, and 120. The presence of a second calcium ion was also reported in the published work of Scott et al. (*22*). This secondary calcium ion was not thought to have a role in catalysis itself, and it was absent in the crystal structure of the wild-type enzyme when a transition state analogue was present in the active site. From this, it was inferred that the binding of calcium at this site might be important in maintaining the surrounding residues in a position favorable for substrate binding (*8*, *22*).

DISCUSSION

In this paper we have described the production and analysis of three active site mutants of the human group IIA $sPLA_2$: H48Q, H48N, and H48A. The H48A was detectable as a 14 kDa band after SDS−PAGE, but no refolded product was obtained. In contrast, the yields of refolded H48Q and H48N were similar to that obtained for the wild-type enzyme. Both mutants expressed significant catalytic activity, and in the case of the H48Q this activity was considerable for a mutation involving a key catalytic residue and was a very unexpected result, particularly since the corresponding mutant of the porcine pancreatic enzyme had been reported to have minimal activity (*10*). This result has implications for the catalytic mechanism discussed below. Detailed analysis of the H48Q using a variety of assay conditions established that this activity was a genuine result of the mutation.

Recently, the H48Q mutant of the human group IIA $sPLA_2$ has been used to evaluate the potential role of catalysis in prothrombinase inhibition by this protein. In this case the mutant had only 0.05% of the wild-type activity (*33*), possibly reflecting a product that had incorrectly folded, as we have observed such contaminants after refolding. The contaminants are often difficult to resolve using the heparin or anionic chromatography columns used in the purification of these enzymes.

Catalytic Mechanism. As recently reviewed (*9*), Verheij et al. (*34*) proposed that Asp-99, His-48, and a water molecule form a catalytic triad in which deprotonation of the water molecule by a basic His-48 generates the attacking nucleophile (Figure 6A). This mechanism was analogous to the classical triad mechanism proposed for serine proteases where the active site serine is the attacking nucleophile generating an acyl-enzyme intermediate. Apart from not involving serine, another difference with the $sPLA_2$ mechanism is the fact that the Asp-99 hydrogen bonds to the ϵNH of the histidine (*9*). The *sn*-2 ester carbonyl of the phospholipid substrate coordinates with the active site calcium ion and polarizes it to facilitate the nucleophilic attack by the deprotonated water. A key feature that has emerged from this mechanism is that the rate-limiting step is presumed to be the formation of the tetrahedral intermediate during which δN of His-48 is protonated. The decomposition of the tetrahedral intermediate gives an alcoholate that must be protonated, and in the mechanism this is by δNH^+ of His-48 (*9*).

In an alternative mechanism, the calcium-coordinated oxyanion mechanism (Figure 7A), the overall proposal

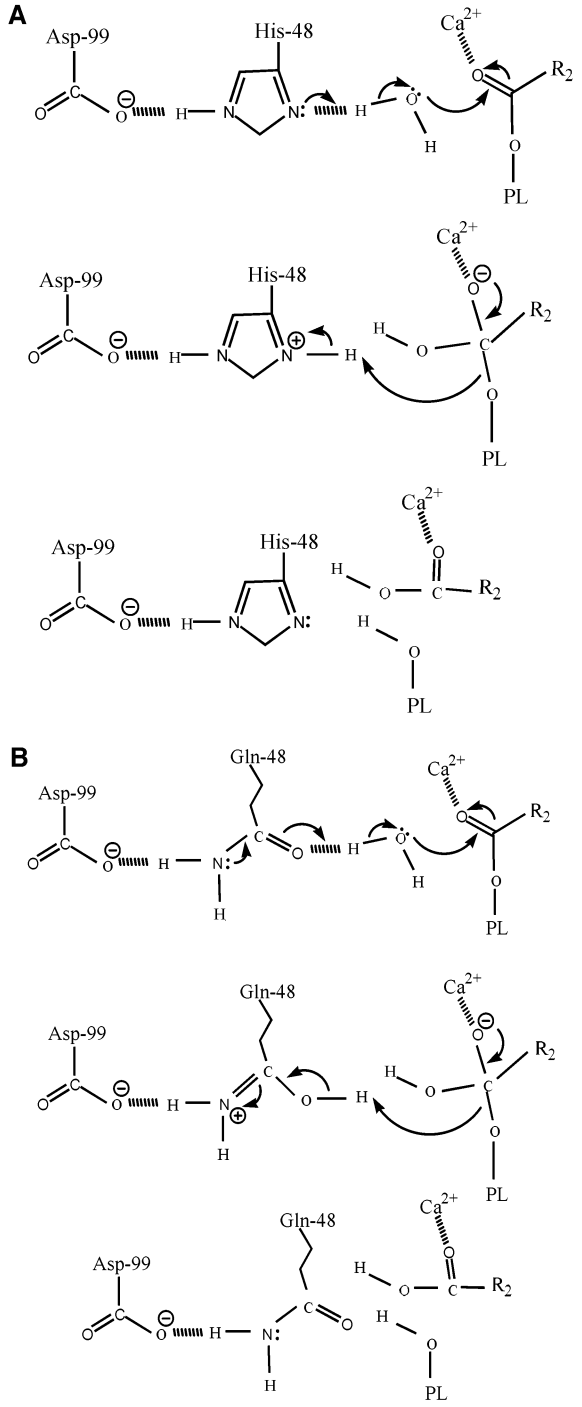

FIGURE 6: Catalytic triad mechanism proposed by Verheij et al. (*34*) for $sPLA_2$. (A) In the Verheij mechanism it is proposed that Asp-99, His-48, and a water molecule (w5) form a catalytic triad in which deprotonation of the water molecule by a basic His-48 generates the attacking nucleophile. (B) Required mechanism for the H48Q mutant in which the proton that is removed from w5 has to be transferred to the carbonyl oxygen of the glutamine side chain. R_2 is the *sn*-2 fatty acid while PL represents the remainder of the phospholipid molecule.

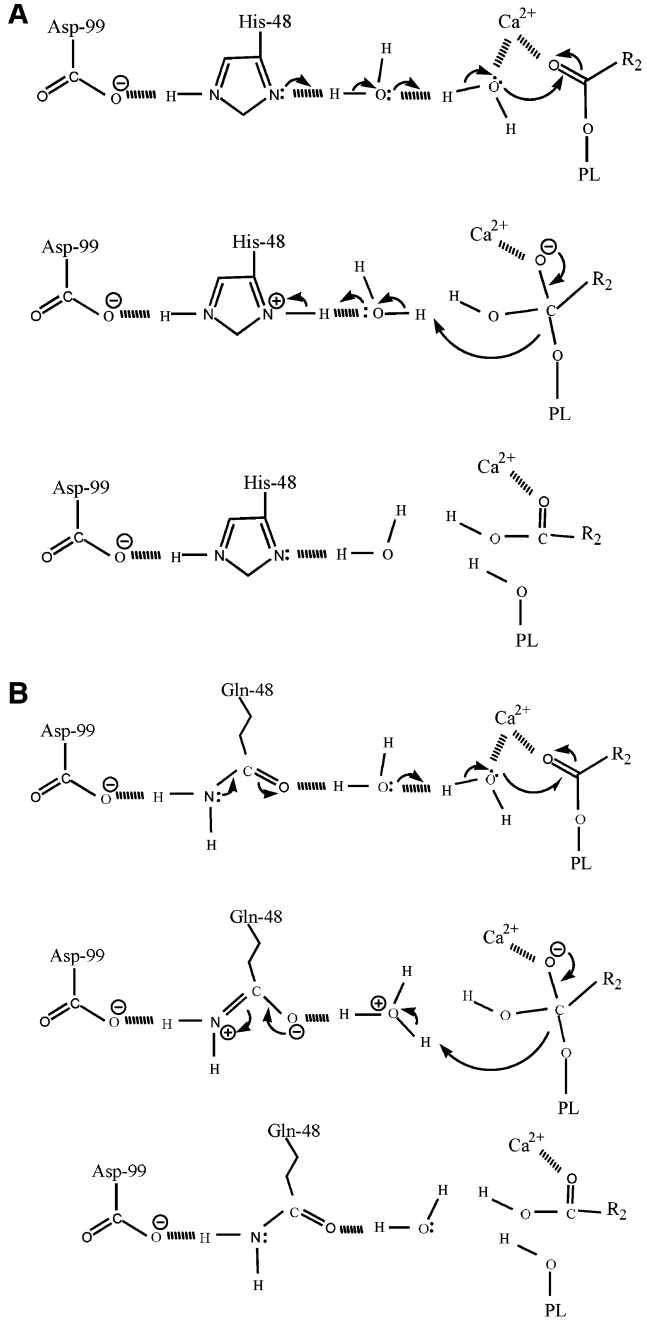

FIGURE 7: Calcium-coordinated oxyanion mechanism proposed by Yu et al. (*28*) for $sPLA_2$. (A) In the Jain mechanism, the water molecule (w5) that generates the attacking nucleophile is coordinated to the calcium ion while w6 is directly linked to His-48. Hydrogen bonding will facilitate electron flow and the resulting proton transfer. (B) Proposed mechanism for the H48Q mutant in which deprotonation of w5 results in protonation of w6. This H_3O^+ is stabilized by the resonance form of Gln-48 that in turn is stabilized via hydrogen bonding by Asp-99. R_2 is the *sn*-2 fatty acid while PL represents the remainder of the phospholipid molecule.

involves *two* water molecules, w5 and w6, in which w5 is coordinated to the Ca^{2+}. The detailed arguments for this mechanism have been discussed elsewhere (*9*, *28*) and is supported by recent structural studies (*35*, *36*). A key feature is that the Ca^{2+}-coordinated w5 will lower the pK_a of this water, a process assisted by the H-bonding to the more basic electron pair of O1 of Asp-49. It is argued (*9*) that a consequence of the oxyanion mechanism is that the rate-limiting step now lies during the decomposition of the tetrahedral intermediate.

The mechanism we would favor to explain the considerable activity of the H48Q mutant is a variation of the oxyanion mechanism, primarily because of the proposed placement of the rate-limiting step beyond the involvement of His-48 as a proton acceptor (*9*, *28*). In this mechanism w6 remains hydrogen bonded to the carbonyl oxygen of Gln-

48, and there is no formal deprotonation of w6, which therefore remains protonated following formation of the oxyanion (Figure 7B). Such proton transfer to w6 will be aided by resonance of the amide bond of Gln-48 that will result in the carbonyl oxygen becoming more negative and thus further stabilizing the positive charge that develops on w6 during catalysis (Figure 7B). Asp-99 will assist in this overall process by stabilizing the positive charge that develops on the amide nitrogen. The subsequent loss of this proton from w6 will accompany protonation of the alcoholate to generate the lysophospholipid product.

In principle, the same basic mechanism could operate using the Verheij mechanism but would require formal protonation of the zwitterionic resonance form of the amide group of Gln-48 (Figure 6B). We would argue that protonation of the amide group of the Gln-48 is energetically less favorable than protonation of w6 as w6 can achieve charge stabilization by multiple hydrogen-bonding interaction through to Asp-99.

An important question is why such a significant residual rate is not achieved with the H48Q mutants of the bovine and porcine pancreatic enzymes (*10*, *11*) where the primary catalytic machinery is identical. Presumably, subtle differences in the active site geometry of these mutants could result in less favorable hydrogen-bonding networks from the Gln-48, making electron flow and proton transfer more difficult. A particular feature of the structure that differs between the bovine pancreatic enzyme and the human group IIA enzyme is the presence of a second Ca^{2+} binding site in the IIA enzyme as discussed above. It is not clear what role, if any, this second Ca^{2+} site plays in catalysis, but it has been proposed that this calcium may act as a second electrophile and assist the primary calcium in neutralizing the oxyanion (*8*, *37*). A second Ca^{2+} binding site is seen in the porcine pancreatic enzyme (*8*, *37*).

Conservative mutations of the active site Asp-99 in the case of the pancreatic enzymes (*38*) such as D99N also produced mutants with significant (~5%) activity. In a manner similar to Gln-48 (Figure 7), resonance of the side chain amide of Asn-99 will help to stabilize the formal positive charge acquired by the His-48 during catalysis. Such a proposal would require the amide oxygen of Asn-99 to be orientated toward His-48 as has been confirmed from the crystal structure of the D99N mutant (*39*). It has been argued (*28*) that the considerable (~5%) activity of D99N compared with the only 0.01% activity for the equivalent D102N mutant of trypsin is consistent with initial nucleophilic attack being rate limiting in the case of trypsin but not for the phospholipase.

In another potential mechanistic variation, it would now appear that naturally occurring Asp → Lys-49 mutants of PLA_2 such as BP-I and BP-II from *Trimeresurus flavoridis* can express significant activity (*40*). Recent crystallographic studies with the Lys-49 protein from *Bothrops pirajai* demonstrate the presence of a free fatty acid at the active site, and it is argued that catalysis may be limited by the rate of product (fatty acid) release from the active site with these proteins (*41*).

Overall, the mechanism of this enzyme appears to demonstrate considerable plasticity as significant changes in key active site residues result in relatively modest changes in catalytic activity. Against this mechanistic background, it is perhaps not surprising that the H48N mutant of the human group IIA enzymes also demonstrates significant enzyme activity.

In summary, the analysis of the catalytic activity of the active site mutant H48Q of the human group IIA enzyme, which has considerable residual catalytic activity, has provided an important insight into the catalytic mechanism of this enzyme.

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