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# Studies of the Roles of High Charge and a Lack of Tryptophan Residues on the Properties of Human Group IIA Secreted Phospholipase $A_2$

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A thesis submitted for the degree of Doctor of Philosophy

Division of Biochemistry and Molecular Biology

November 2002

UNIVERSITY OF SOUTHAMPTON

**Abstract**

FACULTY OF SCIENCE

DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

**Doctor of Philosophy**

# Studies of the roles of high charge and a lack of tryptophan residues on the properties of human group IIA secreted phospholipase $A_2$

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Phospholipase $A_2$ ($PLA_2$) is a large group of enzymes that catalyse the hydrolysis of the sn-2 acyl group ester bond of L-1,2 glycero-phospholipids found in membranes, releasing a free fatty acid and a lyso-phospholipid. Twelve different groups of $PLA_2$ have been identified to date from a diverse range of organisms and with a variety of cellular and extracellular locations. These enzymes are involved in a range of physiological processes including phospholipid digestion and metabolism, signal transduction and host defence.

The subject of this thesis is one of the best studied and characterised of these enzymes the human group IIA secreted phospholipase $A_2$ ($sPLA_2$). The structure of the human group IIA $sPLA_2$ is dominated by two molecular characteristics. Firstly, the enzyme has a very high surface positive charge with an isoelectric point of approximately 10. Secondly, the enzyme does not have tryptophan residues on its membrane-binding surface. The enzyme is an acute phase antibacterial protein with little or no activity on 'self' membranes under normal conditions. The overall aim of this thesis was to establish that the molecular characteristics of this enzyme are responsible for its unique physiological role in the body.

A strategy of charge reversal mutagenesis has been used to show that it is the high global positive charge on the surface of the protein that is required for it to exhibit its potent Gram-positive antibacterial properties. This high charge is responsible for the enzymes' ability to penetrate the bacterial cell wall and gain access to the underlying cell membrane where hydrolysis can take place. A reduction in the overall charge on the enzymes' surface is associated with reduced cell membrane hydrolysis and consequently antibacterial potency.

A strategy involving tryptophan replacement of the non-polar residues within the hydrophobic collar that surrounds the active site entrance has demonstrated that it is the lack of membrane-binding tryptophan residues that enable the protein to exhibit little or no activity on phosphatidylcholine interfaces. The introduction of two tryptophan residues (V3,31W) into the putative membrane-binding surface of the enzyme produced a protein with similar characteristics to the human group V enzyme. These characteristics included the ability of the mutant protein to hydrolyse 'self' membranes and to be internalised into cells. The introduced tryptophan residues have also been used as fluorescence reporter groups to propose a binding model for the protein on the membrane surface.

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# Acknowledgements

I would like to acknowledge the following:

Dr. David Wilton for providing me with the opportunity to work in his laboratory on this project and for his guidance throughout,
BBSRC for funding,
Dr. Andrew Buckland and Dr. Nina Giles for collaboration on work with the tryptophan mutants,
Professor Mike Gelb for the kind gift of charge reversal mutants of hgIIA $sPLA_2$ and for the chance to work in his laboratory,
Professor Wonhwa Cho for advice with respect to $sPLA_2$ internalisation,
and Neville Wright for carrying out mass spectrometry analysis.

Thank you to the members of the Wilton group Andy, Susie, Rob, Jane, Nina and Jo for their good company and poor humour (we never did get that Ferris wheel).

I would also like to thank my family, for their continual support and encouragement in all I have undertaken. A huge thank you must go to my parents for providing me with an upbringing that instilled me with a belief in my abilities, a belief that has helped me to achieve as much as I have. Finally, thank you to my wife Pauline and daughter Amy for helping me to keep things in perspective by reminding me every day of what is truly important.

# Abbreviations

| | |
|---|---|
| BPI | Bactericidal Permeability-Increasing Protein |
| BSA | Bovine Serum Albumin |
| *B.subtilis* | *Bacillus subtilis* |
| CAMP | Cationic Antimicrobial Peptide |
| CMC | Critical Micelle Concentration |
| $cPLA_2$ | Cytosolic Phospholipase $A_2$ |
| DAUDA | 11-(Dansylamino)-undecanoic Acid |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMPC | 1,2-dimyristyl-sn-glycero-3-phospatidylcholine |
| $DO_{et}PC$ | 1,2-di-*O*-octadecenylphosphatidylcholine |
| $DO_{et}PS$ | 1,2-di-*O*-octadecenylphosphatidylserine |
| DOPC | 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine |
| DOPG | 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol |
| DPPC | 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine |
| *E.coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid |
| EPR | Electron Paramagnetic Resonance |
| ESI-MS | Electrospray Ionisation Mass Spectrometry |
| FABP | Fatty Acid Binding Protein |
| FCS | Foetal Calf Serum |
| FPLC | Fast Protein Liquid Chromatography |
| HBSS | Hank's Balanced Salt Solution |
| HEK | Human Embryonic Kidney |
| hg | Human group |
| hgIIA | Human group IIA |
| HSPG | Heparan Sulphate Proteoglycan |
| IBS | Interfacial Binding Surface |
| IL | Interleukin |
| IPTG | Isopropyl-β-thiogalactopyranoside |
| *L.innocua* | *Listeria innocua* |
| *L.monocytogenesis* | *Listeria monocytogenesis* |
| LPS | Lipopolysaccharide |
| LUV | Large Unilamellar Vesicle |
| MAPK | Mitogen-Activated Protein Kinase |
| *M.luteus* | *Micrococcus luteus* |
| MLV | Multi-lamellar Vesicle |
| MOPS | 4-Morpholinepropane sulphonic acid |
| PAF | Platelet-Activating Factor |
| PC | Phosphatidylcholine |
| PCR | Polymerase Chain Reaction |
| PE | Phosphatidylethanolamine |
| PG | Phosphatidylglycerol |
| PI | Phosphatidylinositol |
| $PLA_1$ | Phospholipase $A_1$ |
| $PLA_2$ | Phospholipase $A_2$ |

| | |
|---|---|
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| PMSF | Phenylmethylsulfonyl Fluoride |
| POPC | 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine |
| POPG | 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol |
| PpyrPM | 1-pyrenedecanoyl-2-palmitoyl-sn-glycero-3-phosphomethanol |
| PS | Phosphatidylserine |
| *S.aureus* | *Staphylococcus aureus* |
| $sPLA_2$ | Secretory Phospholipase $A_2$ |
| SPR | Surface Plasmon Resonance |
| SUV | Small Unilamellar Vesicle |
| TNF | Tumour Necrosis Factor |

Standard one and three letter codes for amino acids are used. Secreted phospholipase $A_2$ are numbered according to the sequence alignment of these enzymes with the porcine pancreatic group IB enzyme.

# Chapter One – Introduction

## 1.1 Phospholipases

Phospholipases are enzymes that catalyse the hydrolysis of phospholipids, the major component of cell membranes. There are four classes of phospholipase, $A_1$, $A_2$, C and D, which may be distinguished by the different sites at which they catalyse the hydrolysis of phospholipid molecules, see figure 1.1.

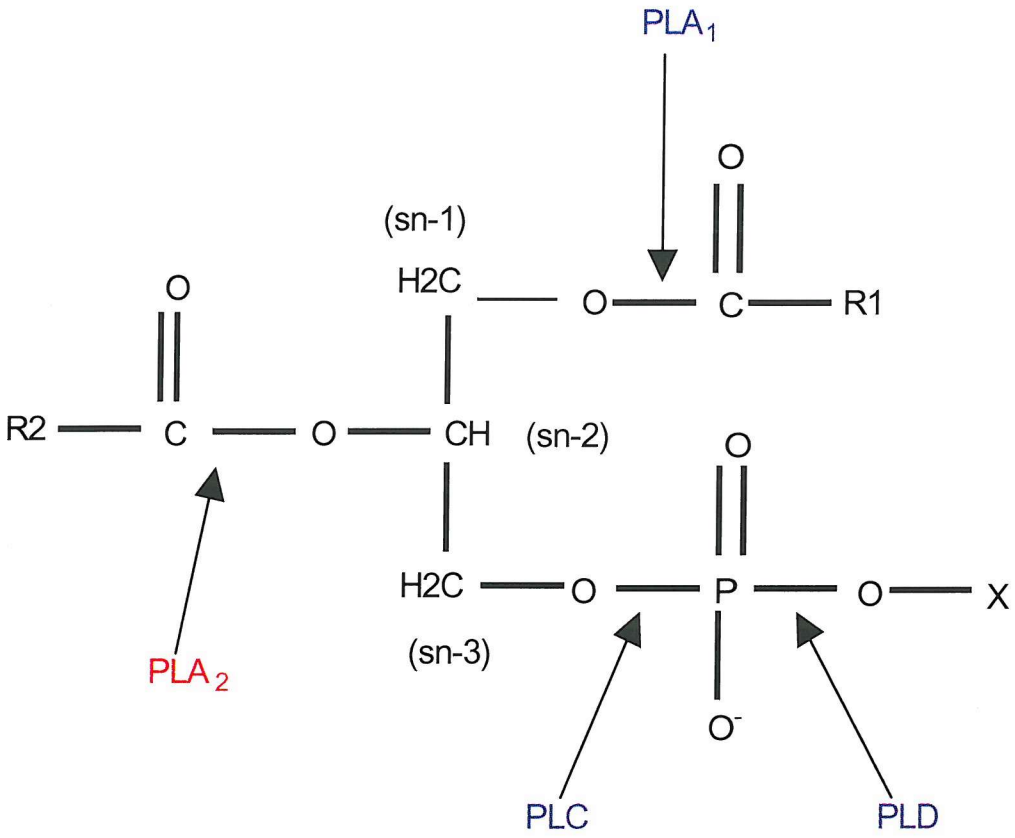


**Figure 1.1** **Sites of catalytic action of phospholipases, $A_1$, $A_2$, C and D**

Arrows indicate the positions of phospholipase catalysed hydrolysis on a glycerol-based phospholipid. R1 and R2 represent the fatty acyl chains and X represents the alcohol head group.

Cell phospholipids have a very low critical micelle concentration (~pM) in aqueous solution (1). Consequently phospholipases have evolved to be directed preferentially against the aggregated forms of these substrates such as vesicles or membranes. Due to the aggregation of the phospholipids there is an increase in the effective concentration of the substrate at the interface and phospholipases that bind to this interface demonstrate what is termed as 'interfacial activation'.

## 1.2 Phospholipase $A_2$

The first identified and most extensively studied of the phospholipase enzymes are the phospholipase $A_2$s ($PLA_2$s). $PLA_2$s cleave the sn-2 acyl group ester bond of L-1,2 glycero-phospholipids releasing a free fatty acid and a lyso-phospholipid.

Many different groups of $PLA_2$ (EC 3.1.1.4) have been identified to date, and these are found in a variety of cellular and extracellular locations. These enzymes are involved in a range of physiological processes including phospholipid digestion, signal transduction and host defence (2;3). The $PLA_2$ super-family has recently been subdivided into two main groups (4), those containing a catalytic histidine residue and those with a catalytic serine, see tables 1.1 and 1.2.

### 1.2.1 Catalytic Histidine $PLA_2$s

Catalytic histidine $PLA_2$s are characteristically small, secreted, contain multiple disulphide bonds and are millimolar $Ca^{++}$ dependent for their catalytic activity.

#### 1.2.1.1 Group I $sPLA_2$s

The cobra venom group IA $PLA_2$ was the first $PLA_2$ to be characterised. The first non-venom $PLA_2$ to be identified was from bovine mammalian pancreatic juices and is classified as the group IB. The group I $sPLA_2$ are characteristic of $sPLA_2$ enzymes. They are small (~13-18kDa), disulphide rich (containing the six absolutely conserved disulphides 27-131, 29-45, 44-109, 51-102, 61-95 and 85-100) (5) and are mM $Ca^{++}$ dependent (6). The group I enzymes were the early models for $sPLA_2$ structure-function analysis providing a wealth of information from x-ray crystal structures and kinetic experiments (7-10). Group I $sPLA_2$s have a characteristic surface loop termed the elapid loop, with the group IB also containing a unique 5 amino acid extension of this loop termed

the pancreatic loop. In addition to the six conserved disulphides they also contain a unique seventh disulphide bond at 11-77. The group IA enzyme is highly active on phosphatidylcholine (PC) interfaces and this fact may reflect the presence on its interfacial binding surface of tryptophan and other aromatic residues which have been shown to partition into the interfacial region of PC bilayers (11-13). The group IB enzyme has a preference for anionic interfaces (14) and exhibits relatively low activity on PC interfaces although this activity can be increased by the presence of anionic lipids in this interface (15). The human group IB enzyme is located on chromosome 12q23-24 (4) and has a clear role in the digestion of dietary phospholipids as well as yet to be defined roles in other physiological processes suggested by its presence in other tissues and the presence of cell surface receptors for this enzyme (3;16).

#### 1.2.1.2 Group II $sPLA_2$s

The group II $sPLA_2$s contain 6 isoforms and the founding members, the group IIA $sPLA_2$s, were initially characterised from the venom of rattlesnakes and vipers. The group was expanded to include the mammalian non-pancreatic enzymes when discovered due to sequence, disulphide bond pattern (unique seventh at 50-138) and loop homologies. The human group IIA enzyme (hgIIA) forms the subject of this thesis and so the group IIA will be discussed later in more detail. Uniquely the IIB enzymes are found only in vipers and are similar to the venom IIA enzymes except they don't contain the otherwise absolutely conserved disulphide bond between residues 61 and 95 (4). Group IIC contains the typical seven disulphides of the group II $PLA_2$s but also an additional one at 87-93 contained within an extended loop region. Originally isolated from the testis of rats, but since seen in brain and pancreatic tissues the IIC is only present in humans as a pseudogene. Group IIC has been found to be active on phosphatidylinositol (PI), PC and phosphatidylethanolamine (PE) (4). The group IID enzyme has been cloned from mouse and humans and detected in the pancreas, spleen and to a lesser degree thymus and colon (17), expressing activity on phosphatidylglycerol (PG), PE and PC with no

obvious substrate discrimination (4). The group IIE enzyme has been identified in both mouse and humans with tissue distribution in mouse testis, brain, heart, liver, uterus and thyroid and in human lung and placenta (17). The group IIE enzyme displays very similar substrate specificity to the IIA enzyme (discussed later). A role for the IIE enzyme in the inflammatory response has been indicated by immunostaining for this enzyme in macrophages in endotoxin challenged group IIA-deficient mice (4). Group IIF is the most recently identified group II enzyme and is expressed in the testes of adult rats and in embryonic tissue (4), the human isoform has only recently been identified (17). The human group II enzymes are all clustered on chromosome 1p34-36 with the closely related human group V $sPLA_2$ (4).

#### 1.2.1.3 Group III $sPLA_2$s

The group III enzyme was originally identified in honeybee venom but isoforms have since been cloned and characterised from a number of different species including human, where it is located on chromosome 22q (17). The enzyme is substantially larger than the other $sPLA_2$s at 55 kDa and is expressed in heart, kidney, skeletal muscle and liver and has a preference for hydrolysing anionic over zwitterionic vesicles (17). The bee venom enzyme has provided a good deal of information about the importance of hydrophobic interactions in interfacial binding and is discussed later in this context (18).

#### 1.2.1.4 Group V $sPLA_2$s

The group V enzymes have no additional disulphide bonds to the six conserved between the $sPLA_2$ groups and in contrast to group IIA the group V enzymes have significant activity against PC and PE (19), an activity associated with the presence of an interfacial tryptophan residue. The group V enzyme in humans is on chromosome 1p34-36 clustered with the group II enzymes with which it is closely related phylogenetically (17). The group V enzymes are found to have a wide tissue distribution and are responsive to

inflammatory stimuli. A role in inflammation and signal transduction has been demonstrated *in vivo* and the enzyme has been seen intracellularly localised to the perinuclear region in close proximity to the inflammatory signalling partners group IV (cytosolic) $PLA_2$ and cyclooxygenase (20).

#### 1.2.1.5 Group IX $sPLA_2$

The group IX enzyme is unique and found only in the venom of a marine snail it has little similarity to the other $PLA_2$s and is composed of two polypeptides joined by disulphide bonds (4).

#### 1.2.1.6 Group X $sPLA_2$s

Identified in humans (chromosomal location 16p12-13.1) and since seen in mice this group shares the six conserved disulphides typical of $sPLA_2$s, however, it also contains the two determinant disulphides previously thought to be specific to the group I and II $sPLA_2$ enzymes (4). Human group X is found in the spleen, thymus, leukocytes and lung endothelial cells and is singly N-glycosylated, the recombinant protein has zwitterionic vesicle activity but is less effective against anionic. Significantly exogenously added group X has been reported to cause the release of arachidonic acid, oleic acid and prostaglandin $E_2$ from human cell lines suggesting a role in inflammation and or signal transduction (21).

#### 1.2.1.7 Group XI $sPLA_2$s

Group XI enzymes have been recently found in plants and have very little homology to the other $PLA_2$s (4).

#### 1.2.1.8 Group XII $sPLA_2$

Group XII $sPLA_2$s have recently been identified using reverse transcriptase-polymerase chain reactions (RT-PCR) in murine type 2 helper T cells in two alternatively spliced forms, a human isoform (chromosomal location 4q25) was also detected by GenBank$^{TM}$ Data Bank search with more than 90% homology to mouse gXII-1 (22). The novel $sPLA_2$ of approximately 21kDa contains 14 cysteine residues like the group IIA enzyme but is predicted to share only two of the usually six conserved disulphide bonds characterising $sPLA_2$s, that is 27-131 and 51-102 (22). No information regarding the tissue distribution of the human enzyme was available at the time of completing this thesis. Notably, recombinant hgXII $sPLA_2$ has recently been shown at very high concentrations to exhibit antibacterial activity against the Gram-negative bacterium *E.coli* the only $sPLA_2$ thus far identified to do so, in the absence of outer membrane permeabilising agents (23).

### 1.2.2 Catalytic Serine $PLA_2$s

Catalytic serine $PLA_2$s are characteristically large, intracellular enzymes with no disulphide bonds or catalytic requirement for $Ca^{++}$.

#### 1.2.2.1 Group IV $PLA_2$s

Group IVA $PLA_2$, frequently termed cytosolic $PLA_2$, was the first serine $PLA_2$ identified and was initially reported in human neutrophils and platelets in 1986. Until this time the group IIA enzyme had been associated with the inflammatory response and the generation of eicosanoid second messengers such as the prostaglandins and leukotrienes. However with the arrival of the group IVA enzyme which is cytosolic in location and specific for arachidonic acid at the *sn-2* position the role of the group IIA enzyme in the inflammatory response was thrown into question (2;24). Since this time the IVA enzyme has

gone on to be shown to be the prime regulator of the levels of these inflammatory agents (25-27).

The group IVA $PLA_2$ enzyme is considerably larger at ~85kDa than the $sPLA_2$s and consists of two domains, the N-terminal C2 domain (a micromolar $Ca^{++}$ dependent phospholipid binding domain) and the C-terminal α/β hydrolase $PLA_2$ domain (28). The C2 domain accounts for the micromolar $Ca^{++}$ sensitivity of the enzyme but this dependence is for translocation of the enzyme from the cytosol to the perinuclear region rather than for the catalytic event itself (25). The group IVA $PLA_2$ is also activated by the phosphorylation of four serine residues (437, 454, 505, and 727) by the mitogen activated protein kinase (MAPK) family of enzymes. The roles of serines 437 and 454 are unclear, as they are not conserved within $cPLA_2\alpha$ from different species, however serines 505 and 727 are conserved and have been seen to be phosphorylated in a variety of stimulated cell types (25).

Phosphorylation of serine 505 has been shown to increase enzymatic activity and may augment $Ca^{++}$ induced arachidonic acid release from cells (25). The method of its action is unclear as in some cell models phosphorylation in the absence of $Ca^{++}$ fails to induce arachidonic acid release. It is possibly due to its position near the linker region of the C2 and catalytic domains that phosphorylation of serine 505 induces a conformational change in the $cPLA_2$ structure making the active site better positioned to access substrate from the membrane's surface (25). The second conserved serine, residue 727, can be phosphorylated by protein kinases A and C, however this phosphorylation does not induce significant $PLA_2$ activity. A role for a further as yet unidentified kinase downstream of MAPK has been proposed as inhibition of p38 MAPK reduces the phosphorylation of 727 together with 505. It has been suggested that phosphorylation of serine 727 may induce association of $cPLA_2$ with the membrane in a $Ca^{++}$ independent way although the physiological relevance of this has yet to be determined (25).

The catalytic domain of the enzyme contains catalytic serine-228 which forms an acyl-serine intermediate characteristic of lipases, with aspartate-549

demonstrated to be the second member of the predicted catalytic triad with the third expected member yet to be identified.

Since the discovery of the IVA enzyme, located on chromosome 1q25, group IVB and IVC paralogs have been identified which are to be found on chromosomes 15 and 19 respectively (4). The group IVB enzyme is ~114kDa with an N-terminal extension and an insertion after the C2 domain and is found ubiquitously, although at considerably higher levels in the pancreas, liver, heart and brain. It shares high homology with the IVA enzyme at its active site but lacks the four serine residues shown to play a role in the activation of the IVA enzyme (4). The group IVC $PLA_2$ is smaller than the other two IV enzymes at ~61kDa and lacks the C2 domain and the concomitant $Ca^{++}$ requirement; it is also prenylated at the C-terminus. The group IVC enzyme is expressed most highly in heart and skeletal muscle (4). All the group IV enzymes exhibit in addition to $PLA_2$ activity varying degrees of $PLA_1$, lysoPLA$_1$ and lysoPLA$_2$ activities and these varying activities together with their differing tissue distributions would suggest both different and multiple physiological roles for these cPLA$_2$s. A variety of fungal phospholipase Bs have also been shown to have homology to the group IVA enzyme catalytic machinery and to be able to hydrolyse acyl and phospholipid substrates as demonstrated by the IVC enzyme, although these enzymes are yet to be classified fully (4).

1.2.2.2 Group VI PLA$_2$s

The group VI enzymes represent the first identified $Ca^{++}$-independent PLA$_2$s and there are two paralogs the VIA and B located on human chromosomes 22q13.1 and 7q31 respectively. The group VIA enzyme is ~85kDa and consists of multiple splice variants with 7 or 8 ankyrin repeats which have varying responses to ATP and include two inactive variants (29). The enzyme is found in a variety of tissues and has both cytosolic and membrane locations. Studies to date have led to the conclusion that the group VIA enzyme is a key housekeeping enzyme involved in phospholipid remodelling

(30) and recent reports have suggested roles in signal transduction (29). The group VIB $PLA_2$ has been identified in heart, skeletal and lymphocyte cDNA libraries although its study is at a very early stage (29).

### 1.2.2.3 Group VII $PLA_2$s

Group VII $PLA_2$s are better known as platelet-activating factor acetylhydrolase (31) and their chromosomal locations are yet to be determined. Platelet-activating factor is a derivative of PC and amongst other things a potent pro-inflammatory agent whose effects are attenuated by the hydrolytic action of the group VII $PLA_2$. Group VIIA $PLA_2$ is ~45kDa, secreted and uniquely for a $PLA_2$ has recently been shown not to be an interfacial enzyme (32). This lack of interfacial activity is supposed to be responsible for its specificity for platelet-activating factor (PAF) and oxidatively-truncated phospholipids from solution as only they are able to be present free in solution to any great degree (32). The group VIIB enzyme is intracellular and ~40kDa in size and has been located in the liver and kidneys where it has been seen to respond to oxidative stresses (4). It is assumed that the group VIIB enzyme plays a role protecting the liver and kidney from oxidation much as the secreted group VIIA does in plasma. It is yet to be shown whether the group VIIB enzyme is able only to access substrates from solution although it has demonstrated similar substrate hydrolysing capabilities (31).

### 1.2.2.4 Group VIII $PLA_2$s

The human group VIIIA and B enzymes are ~26kDa, intracellular and are expressed in the brain as two subunits of the heterotrimeric platelet-activating factor acetylhydrolase Ib. The group VIIIA enzyme has yet to have its chromosomal location determined while the VIIIB is found at 11q23. The group VIII $PLA_2$s are very specific for PAF and their role within the neuronal system is yet to be determined (4).

| Group | | Sources | Size (kDa) | Disulphides (No.) | Unique Disulphides | C-term. Extension (No. Res.) | Molecular Characteristics |
|---|---|---|---|---|---|---|---|
| I | A | Cobra, krait Venom | 13-15 | 7 | 11-77 | None | |
| | B | Mammalian pancreas | 13-15 | 7 | 11-77 | None | Propeptide, 5 residue pancreatic/elapid loop |
| II | A | Human synovial fluid/platelets, rattlesnake venom | 13-15 | 7 | 50-138 | 7 | |
| | B | Gaboon viper venom | 13-15 | 6 | 50-138 | 6 | Lacks disulphide at 61-95 |
| | C | Rat, mouse testes/brain/pancreas (human pseudogene) | 15 | 8 | 50-138, 87-93 | 7 | |
| | D | Human, mouse spleen/pancreas/thymus/colon | 14-15 | 7 | 50-138 | 7 | |
| | E | Mouse testis/brain/heart/liver/uterus/thyroid, human lung/placenta | 14-15 | 7 | 50-138 | 7 | |
| | F | Mouse testes/embryo, human | 16-17 | 7 | 50-138 | 30 | Extra cysteine in C-term extension |
| III | | Bee, lizard, scorpion, human heart/kidney/skeletal muscle/liver | 15-18 | 5 | N/A | N/A | Human form (55kDa) has novel N and C-term domains |
| V | | Mammalian heart/lung/macrophage | 14 | 6 | None | None | |
| IX | | Marine snail venom | 14 | 6 | N/A | N/A | Two polypeptides disulphide linked |
| X | | Human spleen/thymus/leukocyte/ lung endothelial | 14 | 8 | 11-77, 50-138 | 8 | N-glycosylated |
| XI | A | Green rice shoots | 12.4 | 6 | N/A | N/A | |
| | B | Green rice shoots | 12.9 | 6 | N/A | N/A | |
| XII | 1 | Mouse type 2 helper T cells (human GenBank™ search result) | 21 | (14 Cysteines) | N/A | N/A | |
| | 2 | Mouse type 2 helper T cells | 21 | (14 Cysteines) | N/A | N/A | |

**Table 1.1 Catalytic Histidine $PLA_2$s**

| Group | | Sources | Alternative name | Size (kDa) | $Ca^{2+}$ effects | Molecular Characteristics |
|---|---|---|---|---|---|---|
| IV | A | Human neutophils/U937 cells/platelets, rat kidney, RAW 264.7 | $cPLA_2$ α | 85 | <μM Membrane translocation | N-term C2 domain, C-term α/ β-hydrolase, phosphorylation |
| | B | Human liver/pancreas/brain/heart | $cPLA_2$ β | 114 | <μM Membrane translocation | N-term extension, C2 domain plus extension, α/ β-hydrolase |
| | C | Human heart/skeletal muscle | $cPLA_2$ γ | 61 | None | C-term prenylation, α/ β-hydrolase |
| VI | A-1 | $P388D_1$ macrophages, CHO cells | $iPLA_2$-A | 85 | N/A | 8 ankyrin repeats |
| | A-2 | Human B-lymphocytes | $IPLA_2$-B | 88 | N/A | 7 ankyrin repeats |
| | B | Human heart/skeletal muscle/lymphocyte | $iPLA_2$-γ/2 | 88 | N/A | Membrane bound |
| VII | A | Mammalian plasma | PAF-AH | 45 | N/A | Secreted, α/β-hydrolase, Ser/His/Asp triad |
| | B | Human, bovine liver/kidney | PAF-AH (II) | 40 | N/A | Myristoylated, Ser/His/Asp triad |
| VIII | A | Human brain | PAF-AH Ib $\alpha_1$ (subunit of trimer) | 26 | N/A | G-protein fold, Ser/His/Asp triad, dimeric |
| | B | Human brain | PAF-AH Ib $\alpha_2$ (subunit of trimer) | 26 | N/A | G-protein fold, Ser/His/Asp triad, hetero/homodimeric |

**Table 1.2** **Catalytic Serine $PLA_2$s**

## 1.3 Reaction Mechanism of Histidine $PLA_2$s

In this large family of enzymes (sections 1.2.1,1.4 and table 1.1) the invariant catalytic residues are a histidine at position 48 supported by an aspartate at 99 forming a catalytic dyad (numbered according to porcine pancreatic enzyme numbering system). This catalytic machinery bears a marked similarity to the catalytic triad of serine proteases and lipases where in the case of histidine $sPLA_2$s the serine residue is replaced by a water molecule (10).

The identification of the catalytic site residue histidine 48 led Verheij *et al.* to propose the first reaction mechanism for this class of enzyme (33). This initial mechanism was supported in detail by the structural studies of Scott *et al.* (7). They proposed a general base mechanism using three crystal structures from two different $sPLA_2$s, inhibited and uninhibited *Naja naja atra* (group IA) and bee-venom (group IIIA). In this mechanism three water molecules were proposed to be present in the active site in the absence of substrate, two co-ordinating the primary $Ca^{++}$ (one axially the other equatorially) and the third water engaging the Nδ1 of Histidine 48. On substrate binding the two $Ca^{++}$ waters are displaced and the third is activated by proton abstraction by Nδ1 of His 48, leading to a nucleophilic attack on the carbonyl present at the sn-2 ester bond of the substrate. Consequently a tetrahedral intermediate is formed at the sn-2 acyl group ester bond. The oxyanion present is stabilised by the primary $Ca^{++}$ in the active site, which is aided by a second more distant $Ca^{++}$ that acts to hyperpolarise the peptide bond C29-N30 to form a supplemental electrophile. The tetrahedral intermediate then collapses with the donation of the previously abstracted proton present at Nδ1 of His 48, leading to product release.

Subsequently Rogers *et al.* proposed a similar mechanism with two important differences; they proposed firstly that two water molecules, as opposed to one, were involved in the formation (W5) and breakdown (W6) of the intermediate (see figure 1.2), secondly that the rate limiting step was the decomposition of

the tetrahedral intermediate (34). This calcium co-ordinated oxyanion mechanism was reviewed in (35), and supported by the findings of Sekar *et al.* in that paper.

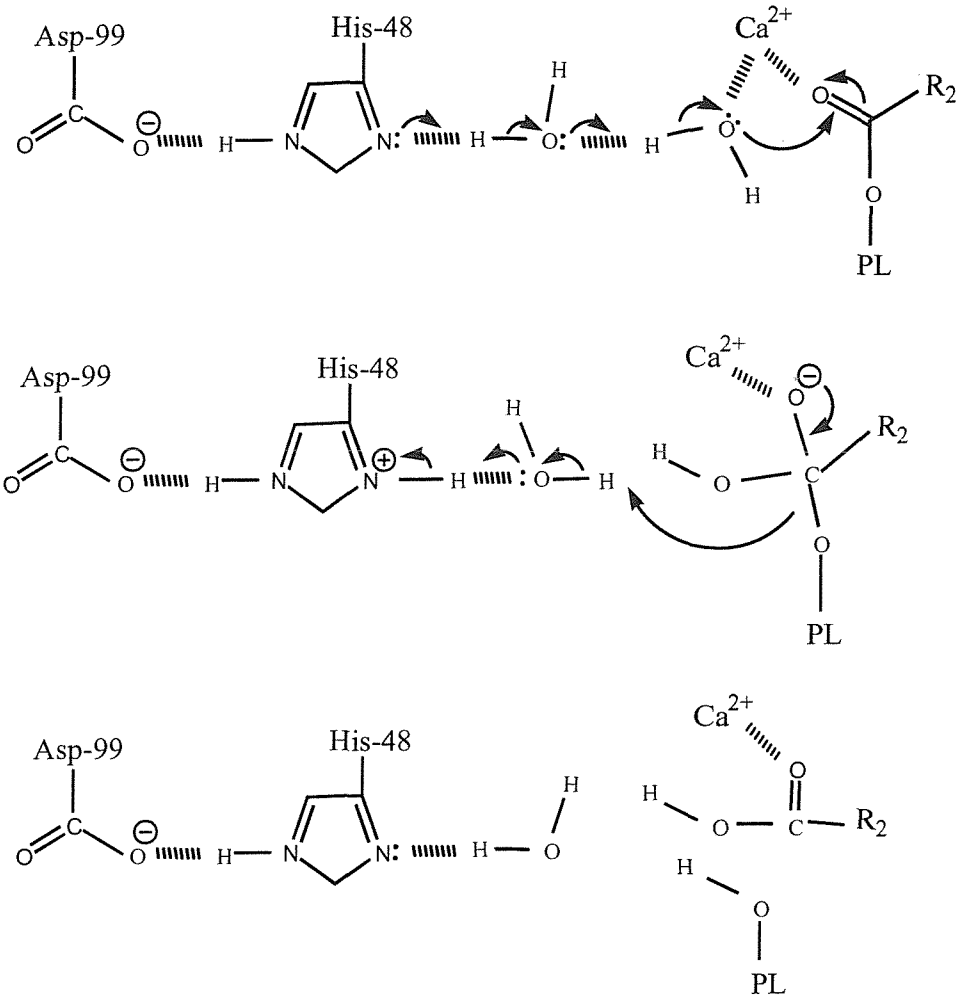


**Figure 1.2** **Proposed $sPLA_2$ reaction mechanism highlighting the role of His 48 and the primary $Ca^{++}$**

R2 represents the sn-2 fatty acid and PL the remainder of the substrate phospholipid molecule. Taken from (36) with permission.

The supporting evidence showed that aspartate 99 in the active site forms a pivotal role in stabilising histidine 48 through its interactions with Nε1 and connecting the catalytic dyad, of which it is a part, to an extensive hydrogen bonding network. This hydrogen bonding network is made up of tyrosines 52 and 73, proline 68, asparagine 71, alanine 1 and glutamine 4 and the structural water W11 (see figure 1.3)

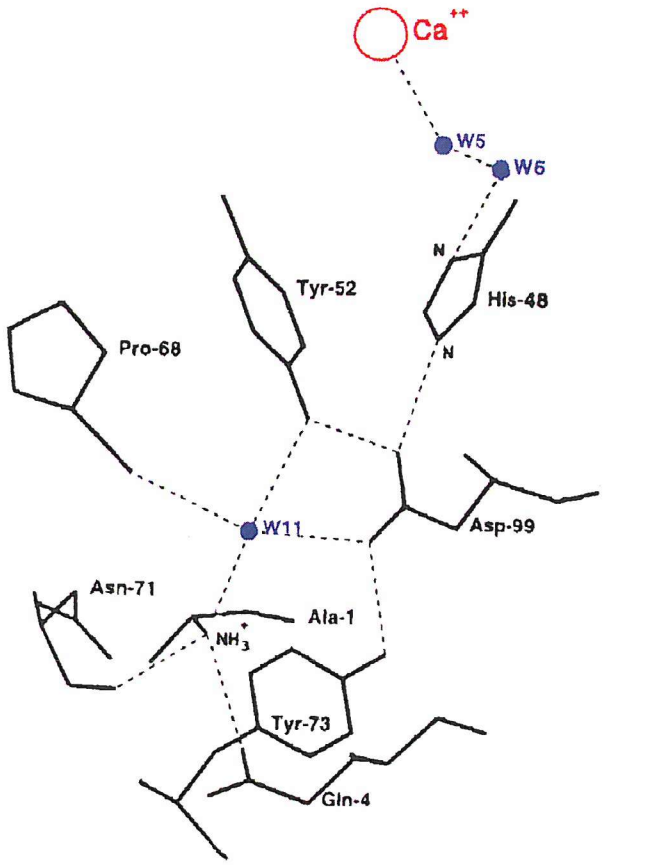


**Figure 1.3** **Hydrogen bonding network of $sPLA_2$s**

Taken from (35) with permission.

The results from kinetic studies of catalytic site mutants also showed that the active site D99N mutation and associated hydrogen bonding network Y52/73N mutant residues did not affect interfacial binding or activation of bovine pancreatic $sPLA_2$ (35). It may be surmised from the close sequence and structural homology of the catalytic apparatus of group I and II enzymes that hgIIA would therefore have the same mechanism. Recent work in this lab, using a catalytically active H48Q mutant of human group IIA (36), has supported this assertion and supports the proposed calcium co-ordinated oxyanion mechanism. It should be noted from the above that $Ca^{++}$ independent interfacial binding and activation are distinct steps from the subsequent $Ca^{++}$ mediated substrate binding and catalysis.

## 1.4 Human group IIA $sPLA_2$

Ten different human $sPLA_2$s have been described to date, nine catalytic histidine $PLA_2$s and one serine (the group VIIA enzyme) as detailed previously (section 1.2), the first of these to be discovered the human group IIA $sPLA_2$ is the subject of this thesis.

Human group IIA $sPLA_2$ (hgIIA) or human non-pancreatic $sPLA_2$ was initially isolated and purified from platelets and the synovial fluid of patients with rheumatoid arthritis (2;37;38) and was the first mammalian non-pancreatic enzyme to be identified. The primary structure of the enzyme bore most homology with group II $sPLA_2$s, as determined by sequence, disulphide pattern, the presence of a small loop between residues 57-68 and a 7 residue C terminal extension. These early predictions have since been borne out by x-ray crystallographic studies (39;40) both in the presence and absence of transition state analogues. The crystal structures have provided a wealth of information regarding the high level of structural homology that the IIA enzyme has with other group II and to a lesser degree some group I $sPLA_2$s. The enzyme has 124 amino acids present as approximately 50% $\alpha$-helix, 10% $\beta$-sheet and the remainder random coil, this isoform has 7 disulphide bonds making it extremely stable and compact, see figure 1.4. In addition to general structural information these crystal structures provided information regarding its reaction mechanism which it is supposed to share with catalytic histidine $sPLA_2$s generally, see section 1.3.

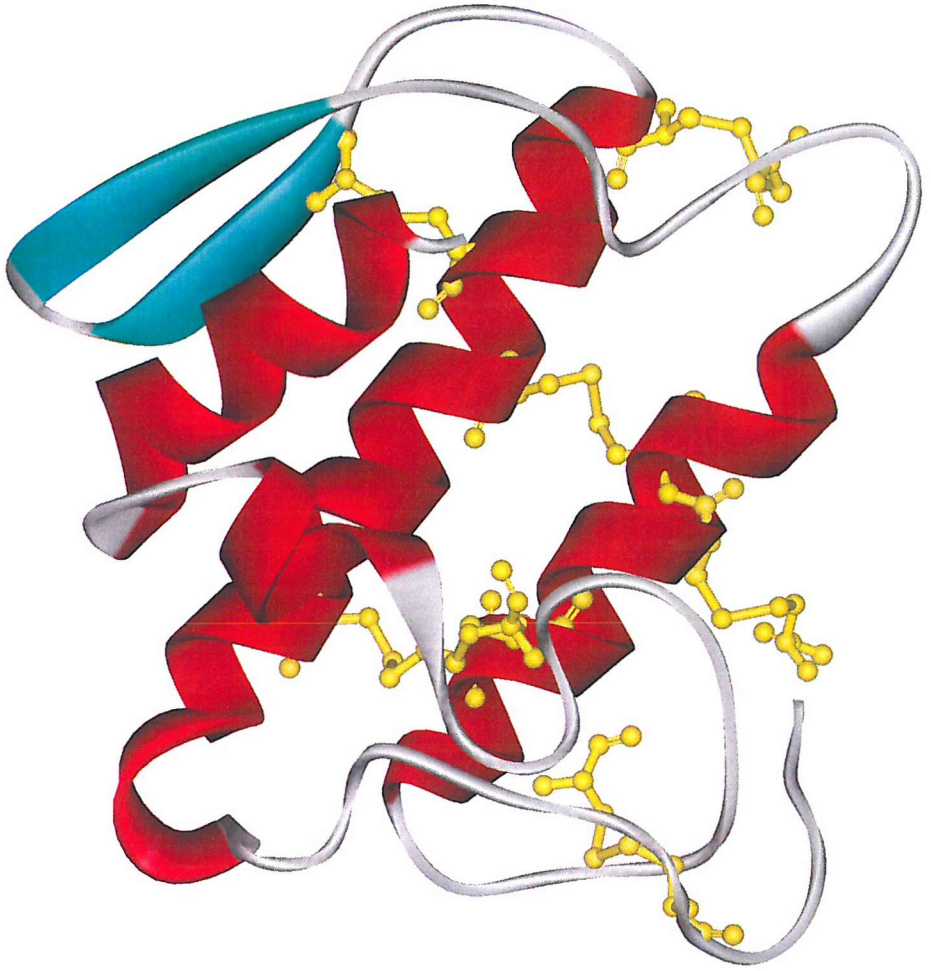

**Figure 1.4** **Ribbon diagram of the structure of human group IIA $sPLA_2$**

Red – α helix, cyan β sheet, grey – random coil, yellow - disulphide forming cysteine residues.

The structural properties of the human group IIA $sPLA_2$ are dominated by two characteristics; a highly positive surface charge due to a large number of cationic residues (ten arginine and thirteen lysine) distributed across the surface of the protein, see figure 1.5, that produce an enzyme with an isoelectric point of approximately 10.5 (37). The second defining characteristic is a high affinity for anionic interfaces and a lack of affinity for zwitterionic interfaces such as those present on the outer leaflet of the plasma membrane (41). These two characteristics it has been suggested are responsible for the physiological characteristics and therefore role of the enzyme, namely as an acute phase anti-bacterial protein with little or no activity on 'self' membranes

under normal conditions. The contributions and molecular basis of these characteristics form the basis of this thesis and will be discussed more fully later.

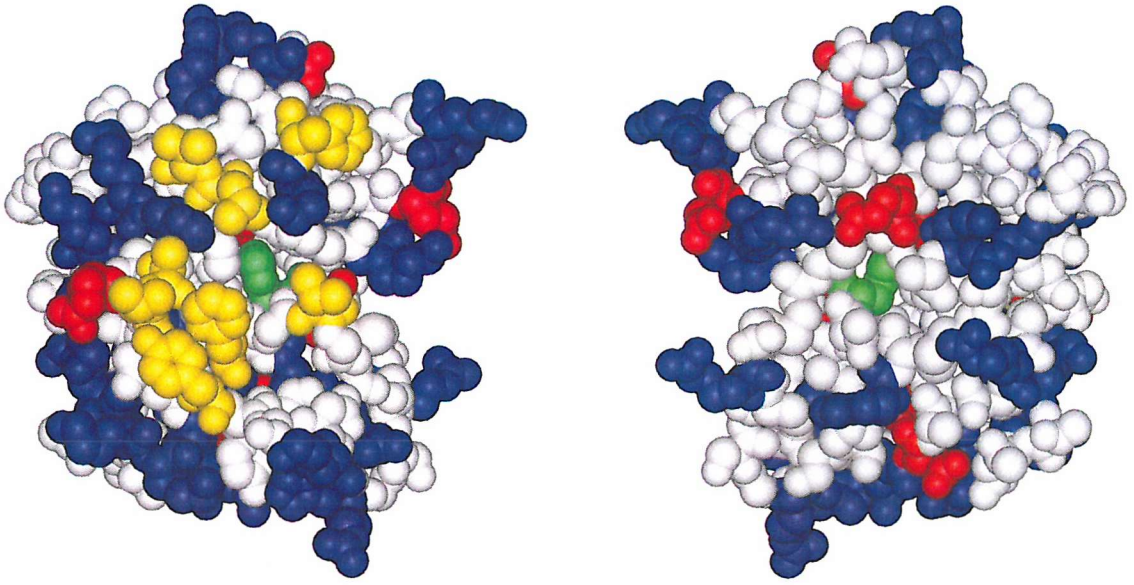

**Figure 1.5** **Space filling representation of hgIIA $sPLA_2$ showing position of cationic residues**

Cationic residues are represented as blue, anionic residues red, hydrophobic residues yellow and the catalytic site histidine residue in green. The image on the left is viewed with the interfacial binding surface pointing towards the viewer and on the right the opposite face with the enzyme rotated about the vertical axis by 180°.

As stated previously (section 1.1), $PLA_2$s are directed preferentially against phospholipid aggregates such as vesicles or membranes therefore interfacial binding is an absolute requirement for catalysis (1). It is the residues constituting the interfacial binding surface, variant amongst different isoforms, which are believed to be largely responsible for the specificity and hence different biological functions of the various $sPLA_2$s, reviewed in section 1.2.1 and table 1.1 (4;12;17). The factors determining these preferences and the relative importance of the various electrostatic and hydrophobic components to the variable binding seen with particular phospholipid interfaces are the source of much interest and is dependant on aspects of both interface and interfacial binding surface, reviewed in (5;42). In the case of the hgIIA $sPLA_2$, the putative interfacial binding surface contains a ring of cationic and

hydrophobic residues (7) and has an exceptionally high affinity for anionic surfaces ($K_d$ ~$10^{-10}$M) whilst exhibiting poor affinity for zwitterionic interfaces (41).

Human group IIA $sPLA_2$ contains a catalytic slot, which extends from the flat surface on one side of the enzyme to the opposite face, see figure 1.5. Previous studies using a variety of methodologies have shown that this flat surface surrounding the opening of the active site slot constitutes the interfacial binding surface (43), and this surface has been suggested to seal the enzyme to the interface in a manner analogous to a suction cup (8). Molecular modelling has suggested that residues L2, V3, A19, L20, F24, V31, F70 and Y119 make up the hydrophobic collar around the active site, see figure 1.5, and are in direct contact with and cause a change in the dielectric constant of the environment surrounding phospholipids in the membrane interface (44). This change in the environment facilitates the diffusion of the substrate phospholipid molecule into the active site, increasing the catalytic efficiency of the enzyme a phenomenon known as interfacial activation (5;7).

In collaboration, the groups of Gelb, Cho and Wilton produced a series of charge reversal mutants of the cationic residues on hgIIAsPLA$_2$ to determine which were important for interfacial binding, see Chapter 3 figure 3.3. It was found that an amino-terminal patch R7/K10/K16 made the most significant contribution supplemented by other groups notably K74/K87/R92 and K124/R127. Despite large changes to the binding affinities, once the phospholipid vesicle concentrations were increased to a level where all enzymes were bound it was found that the catalytic activity of these mutants was comparable to the native hgIIA $sPLA_2$ (41).

Studies showed that electrostatic interactions from residues constituting the interfacial binding surface contribute to the binding affinity of anionic vesicles and that those residues closest to the active site slot made the most significant contribution (41). Notably, they suggested that the low residual binding affinity shown by R7E/K10E/K16E mutant must be due to other cationic residues on the interfacial binding surface and in some part from the hydrophobic residues

that immediately surround the active site slot (the hydrophobic collar discussed above). In support of this assertion subsequent charge reversal mutants were made and R7E/K10E/K16E/K124E/R127D that actually possessed a negatively charged interfacial binding surface still retained significant, if markedly reduced, affinity for anionic vesicles ($K_d$ ~μM).

Further support for the role of hydrophobic residues in the interfacial binding of $sPLA_2$ was provided by studies into the group IIIA honeybee venom enzyme. It was shown that mutating five of the six cationic residues constituting the putative interfacial binding surface had no significant effect on interfacial binding to anionic vesicles and the mode of catalysis of the enzyme (18).

Applying an electron paramagnetic resonance (EPR) and spin label technique it was shown that the bee venom $sPLA_2$ actually contacts the membrane by a prominent patch of hydrophobic residues found on all $sPLA_2$s (45). Further to this it was seen that the cationic residues on this enzyme are not in close proximity to the interface, explaining the lack of effect seen on mutating these residues, and that in marked contrast to the suction cup model the active site opening and substrate are solvent exposed. Although this appears to run contrary to statements made above regarding hgIIA $sPLA_2$ it should be remembered that these are two quite distinct enzymes, with different roles and it should not be surprising if their mode of interfacial binding were different.

The hydrophobic residues, of the group III enzyme, it was suggested "provide a micro-interfacial environment that drives interfacial binding to polar headgroups" (45). It seems that as most $sPLA_2$s show weak binding to zwitterionic membranes but high binding to anionic membranes that these hydrophobic residues are better able to penetrate the latter membranes. Importantly a few $sPLA_2$s, mainly those from snake venom, bind with relatively high affinity to zwitterionic vesicles ($K_d$ ~$10^{-7}$M). These enzymes have 1-3 tryptophan residues on their putative IBS and numerous studies have shown that mutation of these residues have a dramatic effect on affinity (13;46).

There is mounting evidence for the key role of tryptophan residues in lipid-protein interactions. It was shown by Yau *et al.* (11) that it was not amphipathic effects (related to imino group H-bonding or dipole interactions) but rather the flat rigid shape of tryptophan and its aromaticity that favours its insertion into the electrostatically complex interfacial environment; i.e. the region composed of the glycerol backbone and acyl ester bonds.

The dramatic effect that tryptophan residues can have on interfacial binding has been demonstrated with work conducted in this lab (47) where a tryptophan residue replaced a valine in hgIIA in an analogous position to one found within the gIB pancreatic enzyme. The introduced tryptophan was situated in the i-face of the amino-terminal $\alpha$-helix of hgIIA $sPLA_2$ a region shown by charge reversal studies to make the most significant contribution to interfacial binding, see above. The V3W mutant was found to be 200-1000 times more active than the recombinant native enzyme against phosphatidylcholine vesicles and also showed enhanced binding to anionic vesicles as demonstrated by its increased processivity on phosphatidylmethanol vesicles (48).

It is the relative importance and overall contribution of these electrostatic and hydrophobic interactions to the human group IIA $sPLA_2$ membrane binding and physiological function that constitutes the basis of this thesis.

## 1.5 Physiological roles of human group IIA $sPLA_2$

### 1.5.1 Inflammatory

Human group IIA $sPLA_2$ was originally identified from platelets and in the synovial fluid of patients with rheumatoid arthritis (2;37;38). The enzyme has since been found in a variety of tissues such as placenta, macrophages and vascular smooth muscle where it may be present constitutively or when induced by inflammatory cytokines (49). Human group IIA is found to be elevated in serum as a result of trauma and infection where it may aid the removal of damaged tissue, as it can be seen to release arachidonic acid from phospholipids it was suggested to be an important regulator of inflammation. Subsequent to its identification as a possible key regulator in inflammation an 85kDa cytosolic $PLA_2$ was discovered and cloned which was intracellular and specific for arachidonic acid at the sn-2 position (group IV $PLA_2$, section 1.2.2.1). This $cPLA_2$ has since been shown to be the crucial intracellular enzyme mediating the inflammatory response (25-27).

Evidence for the lack of a major role for hgIIA $sPLA_2$ in mediating the inflammatory response comes from studies with hgIIA transgenic mice (50;51) on a null background (52). Mice without $sPLA_2$ showed similar phenotypes and responses to inflammatory challenge to those expressing hgIIA $sPLA_2$ and wild type mice, suggesting a further $sPLA_2$ could compensate for the loss of the enzyme or that it had no major role under these conditions. It has been shown that gIIA $sPLA_2$ is involved in generation of lipid mediators of inflammation (53) but it is now believed that it is pro-inflammatory in the presence of an existing background inflammation and may have a role in disease severity and prolongation.

### 1.5.2 Antibacterial

Human group IIA $sPLA_2$ was shown by Weiss *et al.* (54) to contribute to digestion of ingested Gram-negative bacteria in concert with an activating factor Bactericidal Permeability-Increasing protein (BPI). BPI is an approximately 55kDa cationic protein produced exclusively by precursors of polymorphonuclear leukocytes. The protein is stored in the azurophilic granules of neutrophils and is a member of a group of cationic antimicrobial peptides capable of killing bacteria by oxygen independent mechanisms. The potent cytotoxicity of BPI is restricted to Gram-negative bacteria, reflecting the protein's high affinity for and subsequent complex formation with lipopolysaccharides (LPS) (55). It was shown that hgIIA $sPLA_2$ activity on *E.coli* depended on the presence of amino-terminal basic residues (R7/K10/K16) and that mutations of these residues dramatically reduced its $PLA_2$ activity but that these substitutions had no effect on autoclaved or untreated (-BPI) *E.coli.* The results suggested that a major role of the amino-terminal residues was to mediate electrostatic interactions with the acidic bacterial envelope sites made available by BPI treatment.

A murine type II $sPLA_2$ was isolated as intestinal $sPLA_2$ and demonstrated bactericidal activity against *E.coli* and *L.monocytogenesis.* The enzyme was identified as part of an array of antibacterial factors that protects the upper gastrointestinal tract from microbial invasion (56). It was suggested that the strongly basic (43) group IIA $sPLA_2$s favour interactions with anionic constituents found in bacterial cell walls and membranes. This property it was proposed, could promote the occurrence of high protein concentrations at these sites and that enzymatic activity was required for the antibacterial effect (57).

Weiss and colleagues later showed that the ascitic fluid of sterile inflammatory peritoneal exudates produced in rabbits a potent antibacterial activity against both Gram-negative and Gram-positive bacteria (57). However as seen previously (54) this Gram-negative bactericidal activity was absolutely dependant upon activating factors, in marked contrast Gram-positive activity

against several strains of *S.aureus* was wholly attributable to a Group II 14kDa $sPLA_2$ (57). Anti-rabbit $sPLA_2$ serum was produced that blocked the activity against *S.aureus*, and addition of hgIIA $sPLA_2$ to the serum was found to restore this antibacterial activity. These results showed that the group II $PLA_2$ accounted for the antibacterial properties of the exudate and serum and that these properties were absent from unchallenged plasma.

It has been subsequently shown that hgIIA $sPLA_2$ is the principle bactericidal agent against *S.aureus* and other Gram-positive bacteria (including *M.luteus*) in human tears, despite the presence of lysozyme and lactoferrin in much higher concentrations (58). This potent activity is absent against Gram-negative bacteria (including *E.coli*) in vitro, however as discussed above this activity was previously shown to require activating factors such as BPI (54).

Baboons subjected to a systemic bacterial challenge in the form of a lethal dose of *E.coli* produced a systemic inflammatory response that resulted in the expression of potent antibacterial activity in the plasma of these animals toward both Gram-positive and Gram-negative bacteria (59). This antibacterial activity for Gram-positive bacteria mirrored the over 100 fold rise in group II $sPLA_2$ and was enhanced a further 10 times by factors that were seen to be constitutively present in plasma. The Gram-negative response however was entirely dependent on the action of other antibacterial factors that accumulated in challenged plasma and were not present in pre-challenged serum. Notably the Gram-positive antibacterial response was paralleled by increased bacterial phospholipid degradation.

The kinetics of $sPLA_2$ accumulation was consistent with the notion of it being an acute phase reactant. Moreover, the promoter region of the gene contains several elements homologous with consensus sequences for binding of transcription factors such as activator protein-2, nuclear factor IL-6 and nuclear factor κ-B that are associated with other acute phase genes (60;61). The $PLA_2$ enhancing activity of plasma is specific for live/intact bacterial targets suggesting the action of agents that modify the bacterial cell wall and

thereby facilitate enzyme access and action against cell membrane phospholipids.

Using inbred mice lacking $sPLA_2$ it has been shown that $sPLA_2$ may influence gastric epithelial response to *Helicobacter* infection playing a role in regulating the inflammatory and proliferative responses of the upper gastro-intestinal tract (62). In this study they noted that the $sPLA_2$ null mice showed much more severe inflammatory and hyperplastic responses and that the $sPLA_2^+$ mouse strain exhibiting the highest expression of $sPLA_2$ showed the least response.

It has since been shown *in vivo* using hgIIA $sPLA_2$ transgenic mice on a null background that the expression of this $sPLA_2$ improves resistance against *S.aureus* infection. A response in serum hgIIA $sPLA_2$ levels was also noted after administration of recombinant human IL-1, IL-6, and TNFα (well-characterised components of non-specific host resistance against bacterial infections) supporting the supposed role of these cytokines in activating $sPLA_2$ *in vivo* (63). Subsequently and despite a lack of any real activity *in vitro* in the absence of BPI or complement, resistance was shown in these transgenic mice to *E.coli* infection. It has been suggested that $sPLA_2$ may propagate inflammatory action by activating T-lymphocytes through production of inflammatory mediators, and that it is important in the adhesion of polymorphonuclear leukocytes to tissues (64). Thus the increased resistance to *E.coli* infection seen may result from increased inflammatory response and improved action of leukocytes.

The large and ever expanding number of different mammalian isoforms of $sPLA_2$ make assignment of activity complex. It would seem at this time that the primary role of hgIIA $sPLA_2$ is as an acute phase antibacterial protein and that other isoforms may play roles in arachidonic acid release. Particular examples include the recently identified group V and X $sPLA_2$s (sections 1.2.1.4 and 1.2.1.6 respectively), which unlike hgIIA $sPLA_2$ are active on the outer leaflet of mammalian cells.

## 1.6 Aims

The overall aim is to identify the molecular basis of interfacial binding of human group IIA $sPLA_2$ to phospholipid membrane interfaces. This approach is part of a strategy to explain the known anti-bacterial properties and potential pro-inflammatory role of this enzyme.

Interfacial binding is a balance between electrostatic and non-polar interactions and will reflect the type of membrane to be hydrolysed i.e. the physiological role of the enzyme.

The first objective is to investigate anionic interfaces and the role of cationic residues on the surface of the enzyme in promoting electrostatic interactions and promoting catalysis of Gram-positive bacterial membranes leading to cell death.

The second objective is to investigate zwitterionic interfaces and the role of aromatic residues, particularly, tryptophan in promoting non-polar interactions and catalysis.

# Chapter 2 - Materials and Methods

## 2.1 Materials

Chemicals were purchased from Sigma Aldrich Ltd (Poole, Dorset) and BDH (Poole, Dorset) except for the following specialist items:

Avanti Polar Lipids (Alabama, USA)
1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)

BioRad (Hemel Hempstead, Herts)
Bradford Protein Assay Reagent

Boehringer Mannheim UK Ltd (Lewes, East Sussex)
Restriction Enzymes Nde I, Hind III, Pst I and Buffers

Gibco BRL (Uxbridge, Middlesex)
1Kb Plus DNA Ladder

Molecular Probes (Oregon, USA)
11-(Dansylamino)-undecanoic Acid (DAUDA) and 1-pyrenedecanoyl-2-palmitioyl-sn-glycero-3-phosphomethanol (PpyrPM)

MWG Biotech (Ebersberg, Germany)
PCR Primers oligonucleotides

Novagen (Wisconsin, USA)
Expression plasmid pET11A

Oswel (Southampton, Hants)
PCR Primers oligonucleotides and sequencing

Qiagen (Crawley, West Sussex)
Miniprep Kit, QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit, and QIAquick Nucleotide Removal Kit

Roche Diagnostics Ltd (Lewes, East Sussex)
Restriction Enzyme Pst I and Buffer

### 2.1.1 Bacterial Strains

National Collection of Industrial and Marine Bacteria Ltd (Aberdeen)
*Micrococcus luteus*-strain 9278, *Staphylococcus aureus*-strain 12702

National Collection of Type Cultures (London)
*Listeria innocua*-strain 12210

Novagen (Wisconsin, USA)
*Escherichia Coli*-BL21(DE3)

### 2.1.2 Cell Lines

European Collection of Cell Cultures (Porton Down, Wiltshire)
THP-1 Cells (65), HEK293 Cells (66)

### 2.1.3 Human Group IIA Charge Reversal Mutants

Charge reversal mutants of the hgIIA $sPLA_2$ were the kind gift of Professor Michael H. Gelb, Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington, U.S.A.

## 2.2 Culture Techniques

### 2.2.1 Bacterial Cell Culture

Bacteria were maintained as streaks on suitable nutrient agar plates for approximately 1 week at 4°C. Luria Bertani (LB) broth in the absence/presence of antibiotic (50µg/ml ampicillin or carbenicillin) for *Escherichia coli*, nutrient broth (Oxoid) for *Micrococcus luteus*, and brain heart infusion broth (Oxoid) for *Staphylococcus aureus* and *Listeria innocua*. Long term storage of strains as glycerol stocks was achieved by adding 0.9ml of an overnight culture of cells to 0.1ml sterile glycerol in a sterile cryotube, vortexing and storing at –80°C.

### 2.2.2 Tissue Culture

THP-1 cells, a semi-adherent human monocytic cell line (65), were maintained in low-endotoxin RPMI 1640 supplemented with 2mM Glutamine (Life Sciences Gibco BRL), 10% Heat Inactivated Myoclone Foetal Calf Serum (FCS) (Life Sciences Gibco BRL) and 1% Pen-Strep mix (Penicillin/Streptomycin (10000units/10000µg/ml) (Life Sciences Gibco BRL), 2.92g L-glutamine and 10% β-mercaptoethanol). Cells were sub-cultured between 2-9x$10^5$ cells/ml and split in ratios of 1:3 to 1:4 by tapping the flask to detach any adherent cells then removing cell medium and replacing with fresh complete medium.

HEK293 cells, an adherent human epithelial cell line (66), were maintained in low-endotoxin Dulbecco's modified Eagle's medium (DMEM) with 2mM glutamine, 1% Non Essential Amino Acids (NEAA) (Life Sciences Gibco BRL) and supplemented with 10% FCS. The cells were split as sub-confluent cultures (~75%) in ratios 1:2 to 1:3 i.e. seeding at 3-5x$10^4$ cells/cm$^2$. Media was removed from the cells and the adherent cells washed with 1ml of 0.25% trypsin/EDTA before the addition of 4ml of the same solution for approximately

1 minute or until cells had detached. The trypsin was neutralised with 10ml of culture media and the cells counted using a haemocytometer and diluted with pre-warmed culture media as required. The cells were transferred to a fresh flask and allowed to attach over 24 hours and the media changed.

Cells were maintained in $75cm^2$ Nunc flasks under humidified air at $37^oC$ containing 5% $CO_2$. Long term storage of cell lines was achieved by adding 0.5ml of ~$9x10^5$ cells/ml for THP-1 and ~75% confluent HEK293 cells to 0.5ml of cell freeze medium (50% Dimethyl Sulphoxide, 50% culture medium) in a sterile cryotube, gently flicking to mix and storing above liquid nitrogen.

### 2.2.3 Sterilisation

All broth, buffers, growth media, pipette tips and micro-centrifuge tubes were sterilised by autoclaving at $120^oC$ for 20 minutes where appropriate. Heat labile chemicals were sterile filtered using disposable 0.22µm filters (Schleicher and Schuell, Dassel, Germany).

## 2.3 Recombinant Protein Methods

### 2.3.1 Site Directed Mutagenesis using the Polymerase Chain Reaction (PCR) Mismatch Primer Method

Site directed mutagenesis was performed to make tryptophan mutants of hgIIA $sPLA_2$ using the method described by Higuchi *et al.* (67). The method involves three PCR reactions; firstly a forward and reverse reaction that creates two overlapping fragments containing the inserted mutation and secondly one full reaction that combines the fragments to produce the mutated gene.

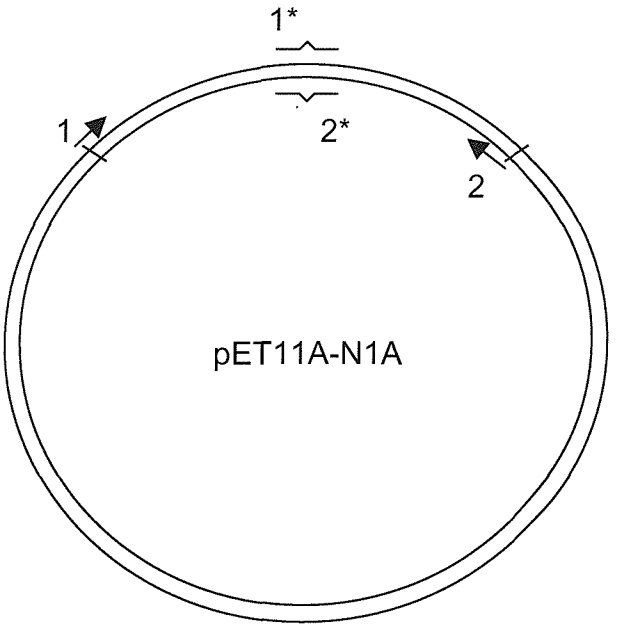


**Figure 2.1** **Schematic diagram of template and PCR primers**

1and 2 are primers complementary to the ends of the gene to be mutated and 1* and 2* are mismatch primers to introduce the required mutation.

2.3.1.1 Oligonucleotide Primers

Oligonucleotide primers used for the construction of the mutants were:
Forward 5' end, G GAT ATA CAT ATG GCC CTG GTA AAC;
Reverse 3' end, CGA TAA GCT TCA CTA TTA GCA ACG;
L2W Forward, A AAA GGA GAT ATA CAT ATG GCC TGG GTA AAC TTC;
L2W Reverse, GAA GTT TAC CCA GGC CAT ATG TAT ATC TCC;
A19W Forward, CC GGT AAA GAA GCC TGG CTG TCT TAC GGT TTC;
A19W Reverse, GAA ACC GTA AGA CAG CCA GGC TTC TTT ACC GG;
L20W Forward, GAA GAA GCT GCT TGG TCT TAC GGT TTG;
L20W Reverse, CA ACC GTA AGA CCA AGC AGC TTC TTC;
F24W Forward, GCT CTG TCT TAC GGT TGG TAC GGT TGC CAC;
F24W Reverse, GTG GCA ACC GTA CCA ACC GTA AGA CAG;
V31W Forward, GT TGC CAC TGC GGT TGG GGC GGC CGC GGG TC;
V31W Reverse, GA CCC GCG GCC GCC CCA ACC GCA GTG GCA AC;
F70W Forward, GC GGT ACC AAA TGG CTG TCT TAC AAA TTC TC;
F70W Reverse, GA GAA TTT GTA AGA CAG CCA TTT GGT ACC GC;
Y119W Forward, C AAC AAA AAA TAC CAG TGG TAC TCT AAC AAA CAC;
Y119W Reverse, GTG TTT GTT AGA GTA CCA CTG GTA TTT TTT GTT G;
Forward$^2$ 5'end, GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG GCC.

The underlined bases indicate the location of the tryptophan mutations in the mismatch primers. The V3,31W double mutant was created using the V3W mutant DNA created previously (47) with the standard Reverse 3' end primer, V31W primers and the Forward$^2$ 5' end primer so as to allow the retention of the V3W mutation. The L2W mutant was created without running the step 1 forward reaction as the mutation was within the area for the forward primer and could be introduced into the forward (sense) strand in the second step by substituting the forward 5' end primer with the forward mismatch primer.

2.3.1.2 PCR Reactions 1 and 2

In the first step, figure 2.2, the forward and reverse reactions are carried out using 125ng of primers (single stranded synthetic oligonucleotides) and 50 or 100ng of template (miniprepped pET11A-N1A expression plasmid). The reactions were catalysed by 3 units of Pfu polymerase, in the presence of 0.2mM dNTPs and Pfu Buffer (20mM Tris-HCl (pH 8.8 at 25°C) 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton® X-100 and 0.1mg/ml nuclease-free BSA).

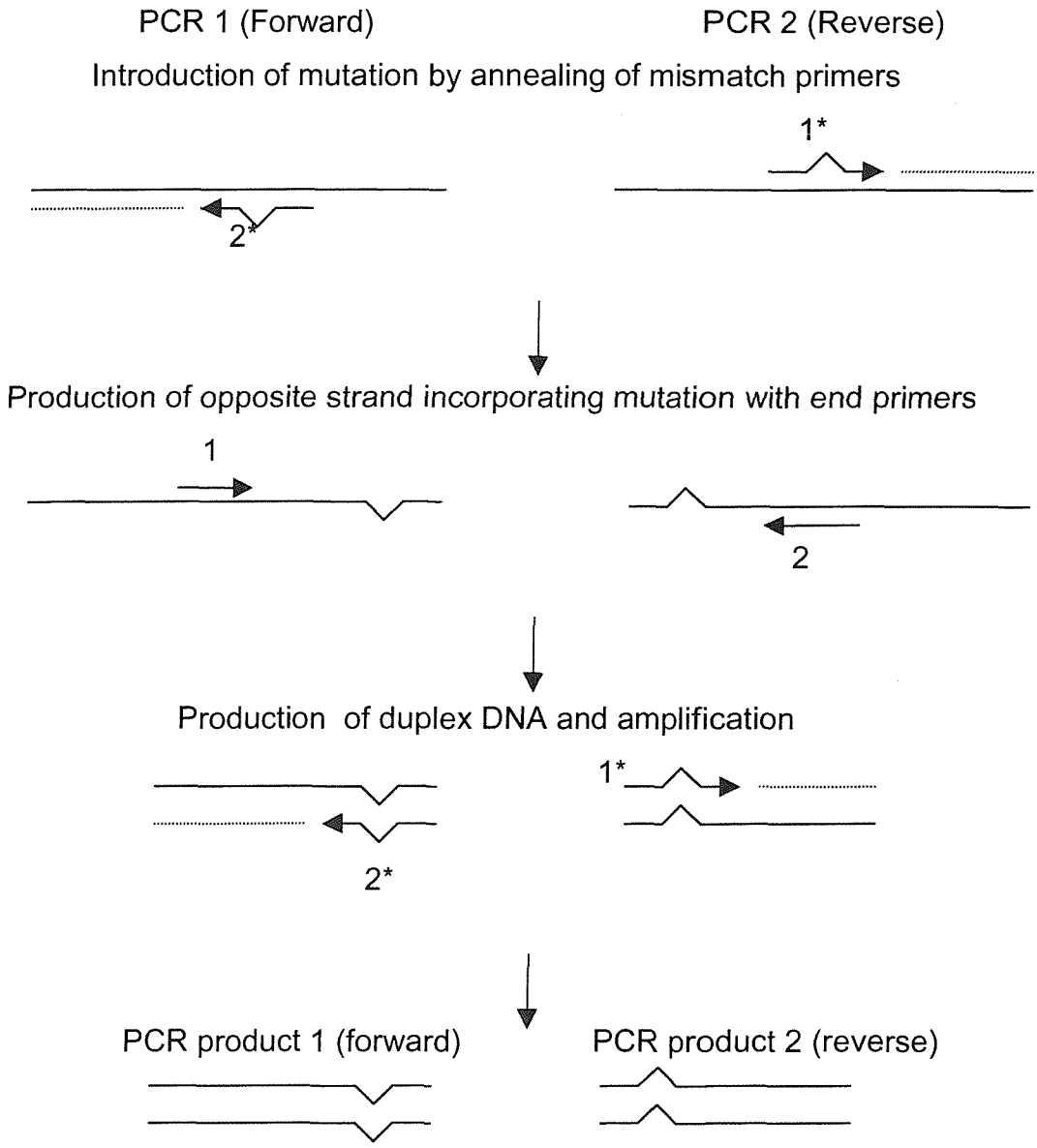


**Figure 2.2** **Schematic diagram of PCR mismatch method step 1**

1and 2 are primers complementary to the ends of the gene to be mutated and 1* and 2* are mismatch primers to introduce the required mutation.

The reactions were carried out using a Techne Progene PCR machine and utilised the following programme;

| | | |
|---|---|---|
| 1. | 94°C 1 minute | Hot start |
| 2. | Added Pfu polymerase | |
| 3. | 94°C 1 minute | Melting |
| | 50°C 1 minute | Annealing |
| | 72°C 1 minute | Extension |
| | 30 cycles | |
| 4. | 72 °C 10 minutes | Amplification |
| 5. | 4 °C | Stop |

PCR products were checked for size and purity by loading 5µl on a 1.5% Agarose gel. The products were then purified using a PCR purification kit and run again on a 1.5% Agarose gel to ascertain the relative quantities of each product.

2.3.1.3 PCR Reaction 3

In the second step, figure 2.3, the full reaction is carried out using 125ng of primers and an equimolar ratio of template (in the form of 2 PCR products from step 1). The reaction is catalysed by 3 units of Pfu polymerase, in the presence of 0.2mM dNTPs and Pfu Buffer using the same programme as for reactions 1 and 2. The PCR product (mutated gene) was analysed for size and purity by loading 5µl on a 1.5% Agarose gel. The product was then purified up using a PCR purification kit and run again on a 1.5% Agarose gel.

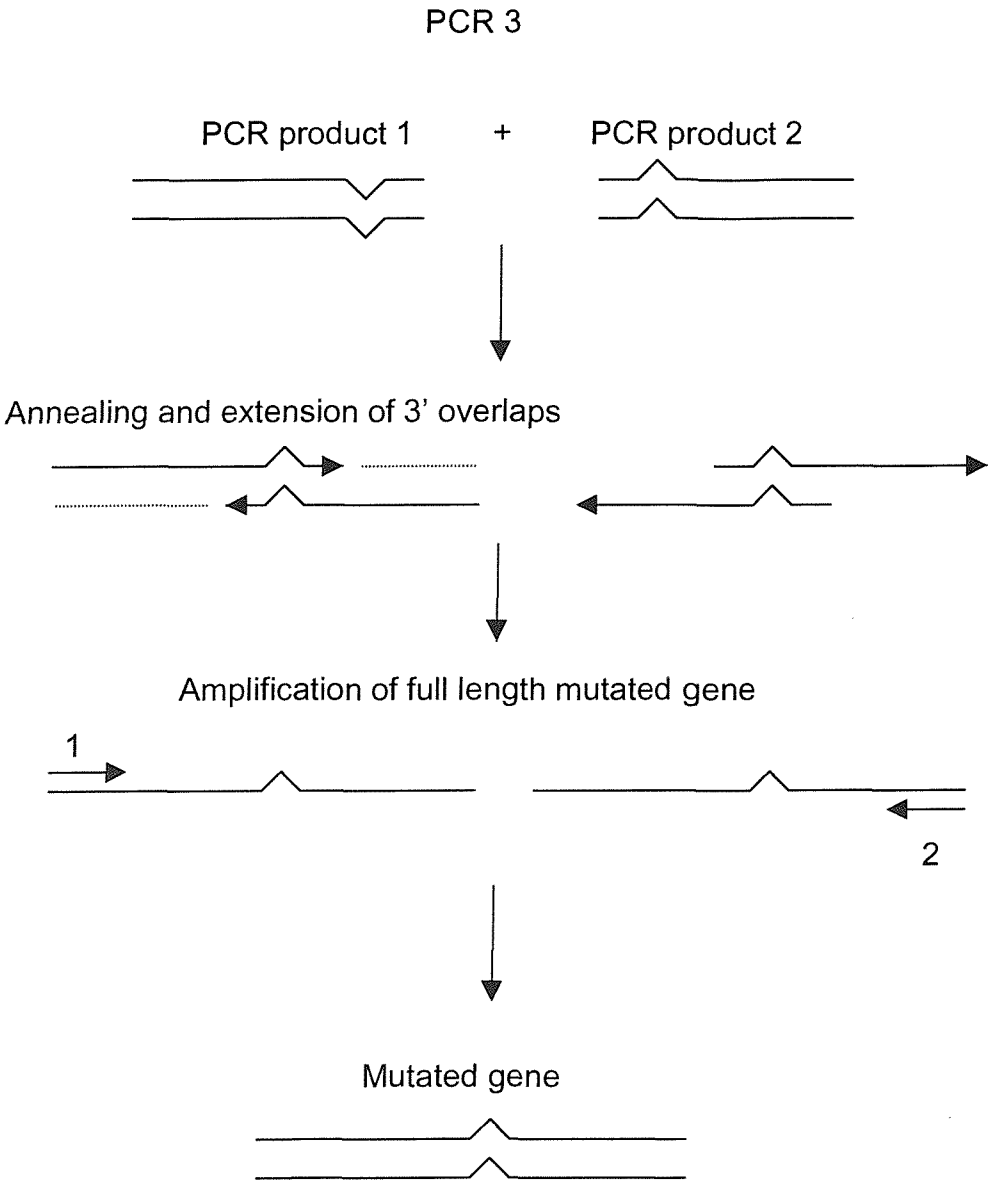


**Figure 2.3** **Schematic diagram of PCR mismatch method step 2**

1and 2 are primers complementary to the ends of the gene to be mutated.

2.3.1.4 Restriction Digest

The mutated gene and target plasmid (miniprepped pET11A) were then digested using Nde 1 and Hind III restriction enzymes in double digests with Buffer B (6mM Tris-HCl (pH 7.5 at 37°C), 6mM $MgCl_2$, 50mM NaCl and 1mM DTT) at 37°C overnight.

The digests (2µl of each) were then run on a 0.8% Agarose gel to check for reaction completion and size of the digested fragments, the PCR digest was then purified using a nucleotide removal kit. The plasmid digest was loaded onto a 0.8% gel and the linearised plasmid isolated using a gel extraction kit. Both of the purified products were then loaded onto a 1.5% agarose gel and checked for purity and relative quantities.

2.3.1.5 Ligation

The digested gene and plasmid were then ligated using T4 DNA Ligase in its buffer (30mM Tris-HCl (pH 7.8 at 25°C), 10mM $MgCl_2$, 10mM DTT and 1mM ATP). The reaction was set up and left in 4l of $H_2O$ at room temperature, which was then placed at 4°C overnight so that the water could cool gradually. The ligation product was then analysed on a 0.8% Agarose gel.

2.3.1.6 Transformation

500µl of an overnight culture of BL21-DE3 cells was diluted into 10ml LB and shaken at 37°C on an orbital shaker, when their $OD_{600}$ was approximately 0.6 the cells harvested by centrifugation at 5620xg for 5 minutes at 4°C. The pelleted cells were resuspended in 5ml of ice cold 50mM $CaCl_2$ and placed on ice for 20 minutes and harvested as before. The pelleted cells were carefully resuspended in 500µl of ice cold 50mM $CaCl_2$. All of the ligation mixture was added to 200µl of cells and left on ice for 90 minutes, before being heat shocked at 42°C for 1 minute and placed back on ice. All of the cells were then plated out onto LB agar plates containing carbenicillin (50µg/ml) left to dry and then inverted and grown at 37°C overnight. Several colonies were selected from the plate and grown in 10ml LB supplemented with carbenicillin (50µg/ml) at 37°C overnight.

5ml of each overnight culture was then miniprepped to give purified plasmid and this was checked for size and purity on a 0.8% agarose gel. 900µl of each overnight was placed into autoclaved cryotubes containing 100µl of sterile glycerol to make glycerol stocks (GS). The miniprepped plasmid was tested for the possible presence of the mutated gene using a restriction digest with Pst 1 in Buffer H (90mM Tris-HCl (pH 7.5 at 37°C), 10mM $MgCl_2$ and 50mM NaCl) at 37°C for 1 hour, and the digestion products run on a 0.8% Agarose gel. Plasmids which when digested produced a cut out fragment in addition to linearised plasmid were then sent for sequencing.

### 2.3.2 Over-expression and Purification of Recombinant Human Group IIA $sPLA_2$

#### 2.3.2.1 Expression of Human gIIA $sPLA_2$

The recombinant human group IIA $sPLA_2$ used throughout this study was produced by R. Othman (47). The gene was constructed by the single step ligation of 12 synthetic oligonucleotides, and was cloned into the pET11a expression vector via Hind III and Eco RI restriction sites which were introduced during the design of the construct. Within the plasmid the gene encoding hgIIA $sPLA_2$ is under the control of the T7 RNA promoter and regulated by the *lac* operon to allow controlled expression of the protein by the addition of Isopropyl-β-thiogalactopyranoside (IPTG). In order that the initiator methionine could be removed by the bacterial amino-peptidase and wild type $PLA_2$ activity expressed, the second amino acid asparagine was mutated to an alanine creating the N1A mutant.

*E.coli* BL21(DE3) cells transformed with pET11A-N1A expression plasmid were grown on sterile Luria Bertani (LB) nutrient-agar (15g/l) plates containing carbenicillin (50µg/ml) at 37°C. Single colonies were selected from the plate and grown overnight in 10ml LB containing carbenicillin (50µg/ml) at 37°C.

Sterile 1l LB broth in fluted flasks were placed at 37°C overnight ready for inoculation. Carbenicillin (50µg/ml) was added to the flasks and two 10ml overnight cultures (ON's) were added to each flask. The cells were then grown on an orbital shaker at approximately 200rpm at 37°C for approximately 3 hours or until $OD_{600}$ is 0.6-1.0. The cells were then induced with IPTG to a final concentration of 0.4mM and grown for a further 5 hours before being harvested by centrifugation at 5620xg at 4°C for 20 minutes using a Beckman centrifuge.

The pellet produced was then resuspended in a minimal amount of resuspension buffer (see below) and stored at -20°C.

2.3.2.2 Isolation and Solubilisation of Inclusion Bodies

The inclusion bodies were isolated using a method modified from that of Bhat *et al.* (68).

The cells were washed in 50ml of resuspension buffer (50mM Tris.HCl pH 8, 50mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA) and 0.5mM phenylmethylsulfonyl fluoride (PMSF)) containing 0.4% (v/v) Triton-X100 and 0.4% (w/v) sodium deoxycholate and stirred for 20 minutes at 4°C. The cell suspension was then sonicated for 15 cycles of 15 seconds with a Misonix XL2020 Sonicator at a power setting of 25% before being collected by centrifugation at 12100xg at 4°C for 10 minutes.

The pellet was then washed again but with 0.8% (v/v) Triton-X100 and 0.8% sodium deoxycholate and then sonicated and centrifuged as before. The pellet was washed a further two times at room temperature, the first with 1% (v/v) Triton-X100 and the second with resuspension buffer alone, the washed inclusion bodies were collected by centrifugation between and after the washes.

The inclusion bodies were solubilised in 25ml of resuspension buffer containing 6M Guanidine.HCl and 5% β-mercaptoethanol stirring at 4°C overnight and the denatured soluble protein was collected as the supernatant after centrifugation at 27200xg at 4°C for 15 minutes. The protein concentration of the supernatant was ascertained using a Bradford assay (see section 2.4.3.2).

### 2.3.2.3 Protein Refolding

The denatured protein was refolded using a method adapted from that of Van Scharrenburg *et al.* (69).

The protein (~0.6g) was refolded at a concentration of <0.15mg/ml by dialysing against 4l of refolding buffer (25mM Tris.HCl pH 8, 5mM $CaCl_2$, 5mM L-Cysteine, 900mM Guanidine.HCl) at 4°C for 72 hours with the buffer renewed every 12 hours. The excess Guanidine.HCl was then removed from the refolded protein solution by dialysing against 4l of dialysis buffer (20mM Tris.HCl pH 7.4, and 2.5mM KCl) at 4°C overnight.

### 2.3.2.4 Purification by SP-Sepharose Cation Exchange Chromatography

A 5ml Hitrap SP-Sepharose column on a Pharmacia Biotech Fast Protein Liquid Chromatogram (FPLC) was loaded with approximately 15 mg of refolded protein at 1ml/minute and washed with 'no salt' Buffer A (10mM Sodium Acetate pH 6) until the absorbance at 280nm was zero. The protein was eluted by a linear gradient (0-100%) of 'salt' Buffer B (Buffer A supplemented with 2M KCl) at 1ml/minute and the fractions collected and assayed for $PLA_2$ content by their absorbance at 280nm and $PLA_2$ activity by using the continuous fluorescence displacement assay (section 2.4.7). Fractions ascertained to contain $PLA_2$ were pooled.

2.3.2.5 Purification by Heparin Sepharose Affinity Chromatography

The pooled $PLA_2$ fractions were diluted 3 times with 'no salt' Buffer C (20mM Tris.HCl pH 7.4) to reduce the KCl concentration prior to being loaded onto a 5ml Hitrap Heparin-Sepharose column at 1ml/minute and washed with Buffer C until the absorbance at 280nm was zero. The protein was eluted by a linear gradient of 'salt' Buffer D (Buffer C supplemented with 1M KCl) with 40% and 100% KCl achieved at elution volumes of 20 and 80ml respectively. The fractions were collected as before and assayed for $PLA_2$ content and pooled. Once pooled the $PLA_2$ was assayed for purity by SDS-PAGE (see section 2.4.5) and the continuous displacement assay (section 2.4.7) to ascertain its specific activity.

### 2.3.3 Over-expression and Purification of Recombinant Rat Liver Fatty Acid Binding Protein (FABP)

2.3.3.1 Expression of Rat Liver FABP

The expression of Rat Liver FABP was carried out as for the human gIIA $sPLA_2$ in *E.coli* BL21(DE3) cells transformed with a pET11A-FABP expression plasmid and harvested in the same manner. The pellet produced was resuspended in a minimal amount of cell lysis buffer (50mM Tris.HCl, pH8.0, 1mM EDTA and 100mM NaCl) and stored $-20^{\circ}C$.

2.3.3.2 Isolation of FABP

The cells were resuspended in 3ml of cell lysis buffer per gram of *E.coli* (assuming 5g/l of culture) containing 4µl of 100mM PMSF and 80µl of 10mg/ml lysozyme per gram of *E.coli* and stirred at $4^{\circ}C$ for 20 minutes. 4mg of sodium deoxycholate per gram of *E.coli* was added and the cell suspension was incubated at $37^{\circ}C$ for 30 minutes, before sonication for 5 cycles of 20

seconds with a Misonix XL2020 Sonicator at a power setting of 25%. The cell debris was then removed by centrifugation at 12100xg at 4°C for 25 minutes and the supernatant kept.

Whilst the supernatant was stirred at 4°C, ammonium sulphate was added slowly to 30% saturation, to remove unwanted proteins. The solution was then stirred for a further hour at this temperature and the resultant precipitate was removed by centrifugation at 12100xg at 4°C for 30 minutes. The ammonium sulphate precipitation was repeated to 60% saturation (FABP is still soluble at this saturation level) and the precipitate again removed as before.

The sample was then dialysed overnight at 4°C against 4l of 0.1M $KH_2PO_4$, pH7.4 to remove the ammonium sulphate. At this time the FABP concentration was estimated using a fluorescence assay.

2.3.3.3 Purification by Dodecyl-agarose Centrifugation

Dodecyl-agarose was shaken to mix and then 2 times 20ml poured into 50ml centrifuge tubes, the dodecyl-agarose was equilibrated and washed 3 times with 20ml of washing buffer (0.1M $KH_2PO_4$, pH7.4) by centrifugation at 12100xg at 4°C for 1 minute. The protein sample was then loaded onto the dodecyl-agarose to allow 10mg of FABP per ml of agarose, 20ml at a time. After each addition the samples were shaken to maximise the binding of FABP to the agarose and then spun at 12100xg at 4°C for 1 minute and the supernatant poured off. When the FABP was fully loaded, as determined by the fluorescence increase of the supernatant, the dodecyl-agarose was washed as before 3 times to remove unbound proteins.

The bound FABP was then eluted from the dodecyl-agarose columns with 20ml of eluting buffer (0.05M $KH_2PO_4$, pH7.4 with 40% ethanol) shaken and then the supernatant collected by centrifugation at 12100xg at 4°C for 1 minute. This elution was repeated with 10ml of buffer until the fluorescence of the eluted samples fell below 150-200 units. The samples obtained with a

fluorescence increase of greater than 200 units were pooled and dialysed against 20mM $KH_2PO_4$, pH7.4 overnight at 4°C to remove any ethanol present.

## 2.4 Analytical Methods

### 2.4.1 Electrospray Ionisation Mass Spectrometry (ESI-MS)

Mass spectrometry was performed on the mutant proteins made using a Fisons VG QUATTRO II quadropole mass spectrometer in electrospray mode and a Micromass LCT™ orthogonal acceleration time of flight mass spectrometer fitted with a nano-Electrospray source. The mass spectrometers were operated in positive ion mode at a source temperature of 80° C with a cone voltage of 43 V. Control, data acquisition and post-processing were performed using Masslynx 3.4 software. Maximum entropy calculations were achieved using Maxent3™. Samples were prepared by dialysing into 10mM Tris.HCl, ph8.0 at 4°C overnight and then concentrating the samples to approximately 200pM/ml using a Millipore Centricon concentrator. Samples were diluted to run with 50% Acetonitrile and 0.2% Formic acid for quadropole and with 50% MeOH and 1% Formic acid for LCT.

### 2.4.2 Circular Dichroism

Comparative secondary structure Circular Dichroism was performed on the mutant proteins using the method of Lee (70) with a Jasco J-720 spectropolarimeter. Measurements were taken between 190nm and 250nm and spectra were averaged from 3 accumulations. Samples were prepared by dialysing (~200μg/ml) into 10mM $KH_2PO_4$, pH8.0 at 4°C overnight and filtering with a 0.2μm filter.

### 2.4.3 Estimation of Protein Concentration

All protein concentrations were carried out in triplicate using a Hitachi U2000 Spectrophotometer.

#### 2.4.3.1 $A_{280}$

Protein concentrations at 280nm were determined using the Beer-Lambert law ($A=\varepsilon cl$) where the 280nm absorption coefficient ($A_{280}^{1\%,1cm}$) was calculated using the Wetlaufer procedure (71) based on the number of Tryptophan, Tyrosine and Cystines within the protein of interest, where

$\varepsilon^{1\%}$ = (10 x (5550 ΣTrp + 1340 ΣTyr + 150ΣCys))/molecular weight

#### 2.4.3.2 Bradford Assay

Protein concentrations were determined using a method based on that of Bradford (72) and utilises the increase in absorption at 595nm when Coomassie Brilliant Blue G250 binds to protein. The dye reagent concentrate was obtained from Bio-Rad and was used according to their instructions.

#### 2.4.3.3 BCA Assay

Protein concentrations were determined using a method based on that of Smith *et al.* (73) and utilise the increase in absorbance at 562nm caused by the biuret reaction, the reduction of Cu(II) to Cu(I) by protein in a concentration dependant manner. The kit was obtained from Sigma and used according to their instructions.

### 2.4.4 Estimation of DNA Concentration

The concentration of DNA samples were calculated using the absorbance at 260nm ($A_{260}$) where 1 absorbance unit = 50μg/ml of double stranded DNA and 33μg/ml of single stranded DNA.

### 2.4.5 Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE utilising the method of Laemmli (74) was used to analyse protein samples using a Biometra vertical gel electrophoresis tank and a Bio-Rad Power Pak 2000. Gels were prepared using a 15% resolving gel (5ml resolving gel buffer (0.75M Tris.HCl, 2ml of 10% SDS, pH8.8), 5ml Protogel (30% acrylamide), 5μl N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and a microspatula of ammonium persulfate (APS)) and a 4% stacking gel (3.75ml stacking gel buffer (0.25M Tris.HCl, 2ml of 10% SDS, pH6.8), 1ml Protogel, 2.75ml $H_2O$, 3μl (TEMED) and a microspatula of APS). The gel suspended vertically in a gel tank containing 1x reservoir buffer (0.1M Tris.HCl, 0.77M glycine, 1% SDS, pH8.3) and samples were loaded in a ratio of 4:1 v/v with sample buffer(2.5ml stacking gel buffer, 4ml of 10% SDS, 2ml glycerol, 0.2ml β-mercaptoethanol, a spatula of bromophenol blue and 1.3ml $H_2O$). The gel was run at a constant 20mA until the dye front was approximately 1cm from the bottom of the gel. The samples were visualised by submerging the gel in stain (0.25% Coomassie Brilliant Blue (R250), 40% methanol and 7% acetic acid) for 1 hour followed by destain (45% methanol and 5% acetic acid) overnight.

### 2.4.6 Agarose Gel Electrophoresis

Agarose submerged gel electrophoresis was used to analyse DNA samples using a horizontal gel tank and a Pharmacia Biotech EPS600 power pack. The

agarose was prepared using 1xTAE buffer (0.04M Tris.HCl, 0.02M acetic acid, and 1mM EDTA, pH7.2) containing 0.6µg/ml ethidium bromide and either 0.8 or 1.5% w/v agarose depending on the size of the DNA to be analysed. The gel was submerged in a gel tank containing 1xTAE buffer with 0.6µg/ml ethidium bromide and samples were loaded in a ratio of 5:1 v/v with gel loading buffer (0.25% bromophenol blue and 30% glycerol). The gel was run at a constant 120V until the dye front was approximately ¾ of the way down the gel. A UV trans-illuminator was used to visualise the DNA samples.

### 2.4.7 Continuous Fluorescence Displacement Assay

Specific activity assays for $sPLA_2$ were carried out using a continuous fluorescence displacement assay described by Wilton (75) under conditions of saturating concentrations of substrate.

The assay utilises the displacement of the fluorescent fatty acid probe 11-(dansylamino) undecanoic acid (DAUDA), see figure 2.4, from rat liver fatty-acid-binding protein (FABP), by long chain fatty acid released as a result of $PLA_2$ catalysed hydrolysis of interfacial phospholipids.

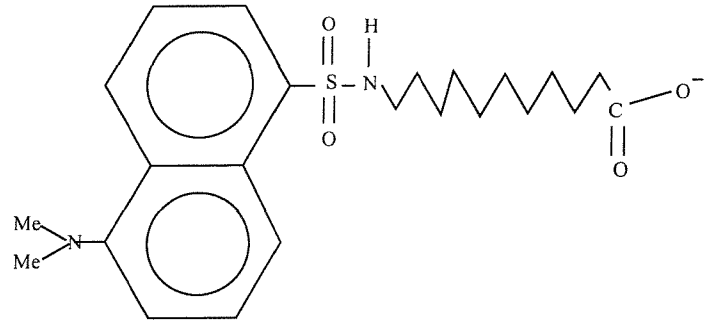


**Figure 2.4** **Schematic of fluorescent probe DAUDA**

DAUDA is relatively non-fluorescent in aqueous solution but highly fluorescent in a non-polar environment into which it will partition due to its hydrophobic nature. Long-chain fatty acids released by $PLA_2$ displace the DAUDA leading

to a dramatic loss in fluorescence, see figure 2.5. The rate of this initial loss in fluorescence is directly related to enzyme activity in a linear way over a 100-fold range in enzyme concentration.

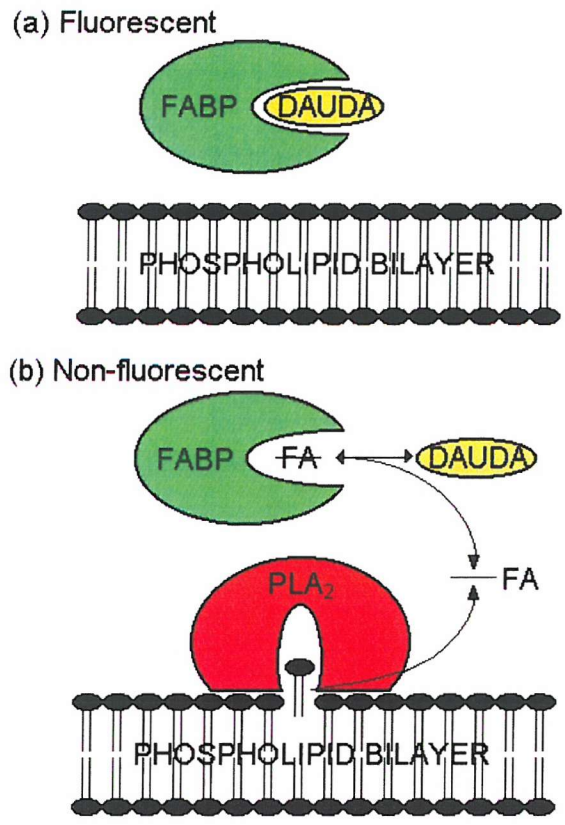


**Figure 2.5** **Cartoon diagram of the fluorescence displacement assay**

The assay is very sensitive ~10pmol/min/ml and has the advantage over comparable systems in being both rapid and applicable to virtually any phospholipid system whether they be present as micelles or whole cells.

2.4.7.1 Preparation of Small Unilamellar Vesicles (SUVs)

Phospholipids were dried down under a stream of nitrogen and then under vacuum for approximately half an hour before being resuspended in methanol to 10mg/ml. The SUVs were formed by direct methanol injection into the sample cuvette with the force of injection providing sufficient mixing.

2.4.7.2 Preparation of Multi-lamellar Vesicles (MLVs)

Phospholipids were dried down as previously described and were resuspended to 2mg/ml in 0.2M Tris.HCl/0.2M NaCl, pH8.0 and left to stand for 5 minutes. The solution was then vortexed for approximately 2 minutes until the phospholipid was fully resuspended.

2.4.7.3 Preparation of Mixed SUVs

Phospholipids were mixed in the appropriate molar concentrations and dried down as previously described, before being resuspended in methanol to 10mg/ml. SUVs were formed as previously described.

2.4.7.4 Preparation of Bacterial Suspensions

Bacterial suspensions were formed from diluted overnight cultures of bacteria and grown up to an $OD_{600}$ of 0.45 and collected by centrifugation at 5620xg for 10 minutes in a Hettich EBA12 centrifuge. The pelleted cells were resuspended to an $OD_{600}$ of 0.11 in Hanks' Balanced Salt Solution. The cell suspension was added directly to the assay cuvette to a concentration of $1x10^7$ cells/ml, $2x10^7$ cells/ml and $2x10^8$ cells/ml for *Micrococcus luteus, Staphylococcus aureus* and *Listeria innocua* respectively.

2.4.7.5 Preparation of Mammalian Cell Suspensions

Mammalian cell suspensions were formed from cells that had been grown in appropriate media and collected by centrifugation at 2500xg for 8 minutes in a Hettich EBA12 centrifuge. The cell pellet was resuspended in 15mls of sterile Hanks' Balanced Salt Solution to wash before being centrifuged again and finally resuspended in 2mls sterile HBSS. The cell suspension was added directly to the assay cuvette to a concentration of $4x10^5$ cells/ml.

2.4.7.6 Method

Experiments were carried out using an Hitachi F2500 fluorescence spectrophotometer set to an excitation wavelength of 350nm, emission wavelength 500nm with slit widths of 5nm and connected to a computer for data logging and analysis. The assay cocktails were set-up with 0.1MTris.HCl/0.1M NaCl, pH8.0 (for vesicles) or HBSS (for cells) containing 1mM $Ca^{++}$ and 1μM DAUDA at 37°C and allowed to equilibrate for approximately half an hour. The substrate phospholipid in the form of vesicles or cells was then added followed by ~0.8μM FABP. The blank was run for 1-12 minutes dependent upon set-up to allow for any background change in fluorescence, before addition of the $sPLA_2$.

The initial rate was measured and the overall loss in fluorescence was monitored from 1 to 10 minutes, more or less $PLA_2$ was added to subsequent assays to produce a reliably measurable initial rate or as required.

Calibrations were carried out with oleic acid. The fatty acid was injected into the assay instead of enzyme in 0.2mM and 0.1mM increments with a 25μl Hamilton up to 1mM and 0.5mM final concentrations for the vesicle and cell assays respectively.

**2.4.8 Bactericidal Assays**

The colony-forming ability of bacteria was used to assess their viability when incubated in the absence or presence of $sPLA_2$ for various times. Incubation mixtures contained 1-2x$10^7$ bacteria/ml in sterile HBSS supplemented with 1mM sterile $CaCl_2$, 50mg/ml sterile BSA and the appropriate amount of $sPLA_2$. Reactions were carried out at 37°C for up to 2 hours. At various time points aliquots were taken and serially diluted into sterile HBSS supplemented with 1mM EGTA to stop $PLA_2$ activity, 10μl samples were then streaked onto

media agar plates and colony forming units determined after 24-36 hours growth at 37°C.

### 2.4.9 Fluorescence spectra

The tryptophan mutants of human group IIA $sPLA_2$ were prepared in 20mM Tris.HCl and 1mM EGTA pH 7.5. All experiments were carried out on a Hitachi F2500 fluorescence spectrophotometer at 25°C, with spectra recorded between 300 and 500nm. Enzyme concentrations of 0.2μM were used and phospholipid SUVs prepared by methanol injection to concentrations of 180μM and 1mM. SUVs were used to minimise light scattering and all measurements were corrected for blank suspensions of wild type (N1A) hgIIA.

### 2.4.10 Fluorescence quenching

All quenching experiments were carried out in 20mM Tris.HCl, 1mM EGTA, pH7.5 at 25°C. Aliquots of 5μl of freshly prepared 3M acrylamide were titrated into 0.2μM protein to a final concentration of 143mM, in the presence or absence of 180μM and 1mM phospholipid SUVs prepared by methanol injection. All experiments were performed on a Hitachi F2500 fluorescence spectrophotometer, excitation wavelength was set at 290nm and emissions recorded between 300 and 500nm or at set wavelengths corresponding to emission wavelength maxima, for wild-type (N1A) blanks and tryptophan mutants respectively.

### 2.4.11 Protein Binding Analysis by Centrifugation of Sucrose-Loaded Vesicles

The binding affinities of the various tryptophan mutants made of hgIIA $sPLA_2$ were investigated using a method (15) based on that of McClaughlin. Solutions containing different concentrations of sucrose-loaded large

unilamellar vesicles and a constant concentration of $sPLA_2$ were prepared and spun down by ultra-centrifugation, the enzyme remaining in the supernatant was then assayed using the PpyrPM fluorescence assay of Radvanyi (76) and the $K_d$s calculated.

2.4.11.1 Preparation of Sucrose-loaded LUVs

Phospholipids were mixed in the appropriate molar concentrations and dried down under a stream of nitrogen and then any trace solvent remaining was removed with a Speed-Vac without heat for 30 minutes. A trace amount of radioactive phospholipid ($^3$H-DPPC, ~90Ci/mmol) was added when the lipid stocks were mixed to allow the tracking of the yield of lipid after extrusion and later the pelleting of vesicles (~400cpm/µl of extrusion solution). The phospholipids were resuspended to a concentration of 30mM with extrusion buffer (176mM sucrose, 1mM 4-Morpholinepropane sulphonic acid (MOPS), pH7.4) and vortexed briefly and frozen in an acetone/dry ice bath. The solution was then thawed completely in a water bath at room temperature and vortexed for 1 minute before repeating the freeze-thaw-vortexing for 7 cycles (ensuring that the solution was frozen and thawed completely each time). At this point the phospholipid solution could be kept at –20°C until required.

A 5µl aliquot was taken and the suspension was transferred to a LiposoFast syringe extruder fitted with two 0.8µm nucleopore membranes (Whatman) and 21 passes carried out. The membranes were then replaced with two 0.1µm membranes and the extrusion repeated for 21 passes and the resultant ~100nm vesicle solution was transferred to a new glass tube. Another 5µl aliquot was then taken and both counted using a Beckman Scintillation counter and the concentration of the vesicle stock calculated by multiplying the concentration of the initial stock by the fractional yield of $^3$H-DPPC.

The vesicle stock was diluted six times, to approximately 4mM total phospholipid, with binding buffer (1mM MOPS, 0.1M KCl, 2mM $CaCl_2$, pH7.4).

The diluted vesicle stock was then spun down in a 1.5ml polyallomer microcentrifuge tube at 100000xg at 21°C for 1 hour using a Beckman ultracentrifuge. The supernatant was removed and replaced by the same volume of binding buffer and the pellet was resuspended gently with a pipette before a 5µl aliquot was taken for scintillation counting so that the final stock concentration could be calculated.

The size and polydispersity of the vesicles were checked using differential light scattering in a Brookhaven Instruments Corp 90Plus Particle Sizer.

2.4.11.2 Binding Reaction

A series of polyallomer tubes were set up into which binding buffer and varying amounts of pre-spun vesicle stock was added to give the correct final concentration of phospholipid, with a final volume of 100µl. To each tube 0.5µg of $sPLA_2$ was added and the solution mixed by gentle flicking, before being spun as before. Once the vesicles were pelleted the supernatant was carefully removed and placed into microcentrifuge tubes on ice. 20µl aliquots were taken and scintillations counted, any reactions where more than 10% of total phospholipid had not pelleted were not included in the final analysis.

2.4.11.3 PpyrPM Fluorescence Assay

Working vesicle solutions of 1-pyrene-2-palmitioyl-sn-glycero-3-phosphomethanol were prepared from a stock solution of 8mM in toluene. The stock solution was dried down under a stream of nitrogen and then any trace solvent remaining was removed with a Speed-Vac without heat for 30 minutes. The phospholipid was then resuspended to a concentration of 64µM with 100µM EGTA. The solution was then vortexed for 1 minute and sonicated for approximately 2 minutes in a bath sonicator until the phospholipid was fully resuspended.

Experiments were carried out using a Jasco fluorimeter set to an excitation wavelength of 342nm, emission wavelength 395nm with a gain of 1 and attenuation of 16 and connected to a chart recorder. 1ml of assay buffer (50mM Tris.HCl, 50mM KCl, 1mM $CaCl_2$ pH8.0) was set-up stirring at 37°C and 20µl of 3% BSA (in assay buffer) was added followed by 20µl of working vesicle solution. The blank was run for 30-60 seconds dependent upon set-up to allow for any background change in fluorescence, before addition of the supernatant containing $sPLA_2$. The initial rate was measured and more or less supernatant was added to subsequent assays to produce a reliably measurable initial rate.

The initial rate was measured for the reaction tube containing no phospholipid and this value was taken as 100%, the initial rates from the reaction tubes containing various amounts of phospholipid were then compared to this and these values plotted versus the total phospholipid present in the binding reaction. The curve was fit to the standard equation for equilibrium dissociation:

$$100 \times (E_F/E_T) = K_d/(L + K_d)$$

Where $E_F$ is the concentration of $sPLA_2$ in the aqueous phase (free), $E_T$ is the concentration of total enzyme (free and vesicle bound) in the binding reaction, L is the total phospholipid concentration in the binding reaction (expressed as total moles of phospholipid, $DO_{et}PC$ + $DO_{et}PS$ divided by the volume of binding reaction sample), and $K_d$ is the equilibrium constant for the dissociation of vesicle-bound enzyme into the aqueous phase.

### 2.4.12 Internalisation of hgIIA $sPLA_2$ treated HEK293 cells

#### 2.4.12.1 Internalisation

Internalisation of hgIIA $sPLA_2$ was carried out based on a method described by Cho and colleagues (20). Mammalian HEK293 cells were grown in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated foetal calf serum. Approximately $1x10^5$ cells/ml were seeded onto Nunc 6 well plates and were grown further until they reached ~75% confluence (~$2x10^5$ cells/ml). The cells were then treated with 100nM of wild type, V3W, L20W, V31W and V3,31W mutants of human group IIA $sPLA_2$ for the times indicated. Incubations were quenched by the addition of a solution of ice-cold 0.6M NaCl supplemented DMEM and then washed in the same solution. The samples were collected by scraping and centrifugation and the resultant pellets lysed in 140µl of cell lysis buffer (20mM Tris.HCl, 30mM $Na_4P_2O_7$, 50mM NaF, 40mM NaCl, 5mM EDTA, pH7.4) containing 1% Triton X-100, 0.5% deoxycholic acid, 1mM phenylmethylsulfonyl fluoride, 2mM $Na_3VO_4$, 10µg/ml leupeptin and 5µg/ml aprotinin. The samples were left on ice for 10 minutes and then centrifuged at 12,000xg for 5 minutes to remove cell debris and the supernatants collected.

#### 2.4.12.2 Western Blotting Analysis

Samples were mixed in a ratio of 5:1 (v/v) with non-reducing sample buffer (0.125M Tris.HCl, pH6.8, 20% (v/v) glycerol, 4% (v/v) SDS, 0.005% bromophenol blue). The samples and control were run on standard SDS-PAGE set-up as detailed (section 2.4.5).

The proteins were transferred from the gel onto a pre-wetted polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer kit (Semi-Phor™, Hoefer Scientific Instruments). The system was run at 45mA for 1 hour with a small amount of transfer buffer (48mM Tris, 39mM glycine, 0.04% SDS in 20%

methanol). The membrane was removed and washed twice with water before being blocked with freshly prepared phosphate buffered saline (PBS) containing 3% non-fat milk protein and 0.1% Tween 20, pH7.4, for 20 minutes at room temperature with constant agitation. The membrane was then incubated with the primary antibody (5μg/ml of mouse anti-human secretory $PLA_2$ IgG) diluted in freshly prepared 3% milk PBS-Tween. The incubation was carried out overnight at 4°C with constant agitation. The membrane was then washed 3 times for 5 minutes with PBS-Tween, before incubation with the secondary antibody (1:10000 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase) diluted in freshly prepared 5% milk PBS-Tween. The incubation was carried out for 1 hour at room temperature with constant agitation. The membrane was then washed 3 times with PBS-Tween and once with water.

Visualisation of the membrane was performed using Supersignal® West Dura Extended Duration Substrate (Pierce, IL, USA) according to the manufacturer's instructions and the membrane was incubated for 15-30 minutes at room temperature. Upon completion of the incubation the membrane was allowed to drip-dry before being placed into a developing cassette. The film (Kodak Biomax MR, Kodak, U.K.) was exposed to the membrane for 5-30 minutes prior to removal and being placed in developer followed by fixer and then washed in water. The film was then analysed for bands that corresponded to hgIIA $sPLA_2$.

Whilst the membrane was prepared the transferred gel was stained and destained, as described previously for SDS-PAGE. When the film had been developed the membrane was placed in stain (0.1% Amido Black, 45% methanol and 5% acetic acid) for ~1 minute and then destained, so that the efficiency of transfer could be examined and the relative transfer of each lane checked.

# Chapter 3 - The role of surface charge in the antibacterial properties of the human group IIA $sPLA_2$

## 3.1 Introduction

Human group IIA $sPLA_2$ is now recognised as expressing antibacterial activity against Gram-positive bacteria, and its presence at high concentrations in inflammatory fluids (38) and human tears along with lysozyme (58) is entirely consistent with this role. The enzyme is also seen to be released from cells which are known to form part of the antibacterial response, that is Paneth cells of the small intestine (56) and macrophages (2;38). The enzyme is unique among the $sPLA_2$ so far studied in that it has almost zero activity against zwitterionic phospholipids as presented in the outer monolayer of the plasma membrane and the monolayer coat of lipoproteins. In contrast, the enzyme expresses high activity against many anionic phospholipids, in particular phosphatidylglycerol. The human group IIA $sPLA_2$ is highly cationic with an isoelectric point of approximately 10.5, due in most part to a large number of positively charged amino acids on the proteins surface. As a result of previous work in this laboratory, it has been suggested that this characteristic is responsible for the antibacterial activity of the enzyme (77).

Humans utilise a variety of factors to kill invading bacteria, ranging from the toxic products of the respiratory burst, products of the complement system, cationic antimicrobial peptides, and lytic enzymes such as lysozyme and gIIA $sPLA_2$. A variety of cationic antimicrobial peptides (CAMP) are produced as part of the innate immune response in all vertebrates. The peptides' site of action is the cell membrane and is due in part to their preference for anionic interfaces (78). There is a vast array of CAMPs produced across the animal and plant kingdoms with little in common except their ability to form structures that are amphipathic in nature, featuring cationic and hydrophobic sections. The mechanism by which they operate is uncertain yet it is known that they insert into the membrane of the microorganism causing disruption and in some way lead to cell death.

It would appear that the highly cationic hgIIA $sPLA_2$, like CAMPs, has targeted a fundamental difference between bacterial and host cellular properties, that is the nature of the charge associated with the exposed surface of the cells. The

outer envelope and membranes of bacteria are dominated by species of molecules that are negatively charged whereas the normal plasma membrane of animals are neutral, producing a fundamental difference that can be exploited by factors that are positively charged.

The outer surface of Gram-negative bacteria consists of the outer cell membrane, see figure 3.1. This membrane which is impermeable to essentially anything but small ions and molecules admitted by the transmembrane porins is anionic due in main to the presence of lipopolysaccharides in its outer leaflet. Lipopolysaccharides are made up of 3 domains; Lipid A, the core subunit and the O-antigen. Anionic Lipid A is composed of a glucosamine dimer with associated phosphate groups and fatty acid chains that forms the outer leaflet of the membrane. The central core subunit is made up of a branched polysaccharide chain with sugars containing 6, 7 and 8 carbons. Connected to the core and stretching out from the membrane is a long carbohydrate chain the O-antigen, whose sequence is very variable and is made up of repeating units of 6 carbon sugars. Lipoproteins and phospholipids are confined in large to the inner leaflet of the membrane. Within the periplasmic space gram-negative bacteria have a thin (1-3 sheets) peptidoglycan layer and below this the cytoplasmic membrane typically dominated by phosphatidylethanolamine and with PG and diphosphatidylglycerol (cardiolipin) present in relatively small amounts.

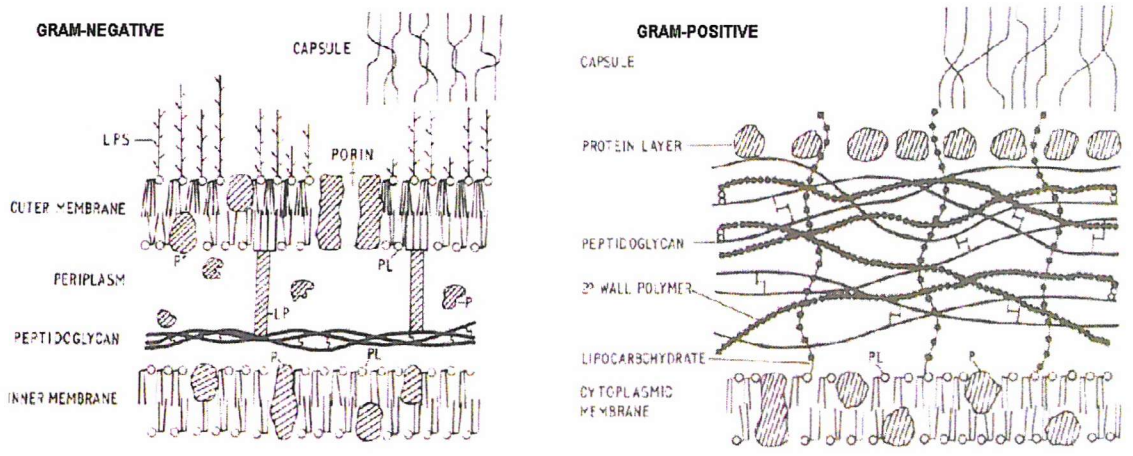


**Figure 3.1** **Structural differences between Gram-negative and Gram-positive bacterial cell walls**

Taken from (79) with permission.

Gram-positive bacteria unlike Gram-negative do not have an outer membrane but possess a thicker highly cross-linked exposed layer of cell wall composed of peptidoglycan, see figure 3.1. Peptidoglycan is a polysaccharide composed of alternating units of N-acetylglucosamine and N-acetylmuramic acid which is cross-linked by L and D forms of amino acids and unique amino acids. As many as 25 sheets of peptidoglycan can be found stacked in the walls of some Gram-positive bacteria and these are connected together by teichoic acid and to the underlying membrane by lipoteichoic acid or by analogous molecules such as succinylated lipomannan (80). These molecules are highly negatively charged and made up of either glycerol or ribitol phosphate polymers in the case of teichoic acids or highly branched α1,6 linked mannopyranosyl groups in the case of lipomannan. The cell wall of Gram-positive bacteria is anionic and highly cross-linked, with the actual thickness of the wall and amount of cross-linking dependant on the type of bacteria and the growth state of the bacteria in question. The cytoplasmic membranes of Gram-positive bacteria are dominated by anionic phospholipids predominantly PG and varying amounts of diphosphatidylglycerol (81).

In contrast to the highly anionic outer surfaces of bacteria, the outer leaflet of the plasma membrane of animals is made up of largely zwitterionic phospholipids (PC and PE) with the majority of negatively charged phospholipids (PS and PG) found on the inner leaflet facing the cytoplasm. High concentrations of cholesterol are also found in the plasma membrane where it resides primarily in the outer monolayer mostly in association with sphingomyelin. The monolayer coat of lipoproteins has a similar composition to the outer leaflet of the plasma membrane.

Bacteria are faced with the vast array of antimicrobial factors produced to defend against their presence and have had to in turn develop mechanisms to protect themselves from attack (reviewed in (78;82)). The mechanisms used are many and various, and it would seem obvious that evolution would have selected for species that had less anionic molecular species in their outer cell walls. However, it seems that such simple single cell organisms are unable to replace their basic requirement for such molecules, due to their requirement

for a membrane potential etc, meaning that they are unable to directly remove the negative charge associated with them. So bacteria both Gram-negative and positive have developed methods to mask their fundamental negativity by a strategy of charge reduction.

Gram-negative bacteria in many cases control the charge on their outer membrane by altering the amount of positively charged ethanolamine and 4-aminoarabinose incorporated into these membranes (82), see figure 3.2. Many Gram-positive bacteria with their thick exposed cell wall of peptidoglycan and (lipo)teichoic acid use a similar strategy of charge reduction. In this case (83) the (lipo)teichoic acid components of their cell wall are esterified with D-alanine, thus reducing the negative charge. In addition to this Gram-positive bacteria may incorporate into their cytoplasmic membrane modified phospholipids such as lysylphosphatidylglycerol a PG derivative with L-lysine esterified onto the headgroup giving the phospholipid a net positive charge, see figure 3.2.

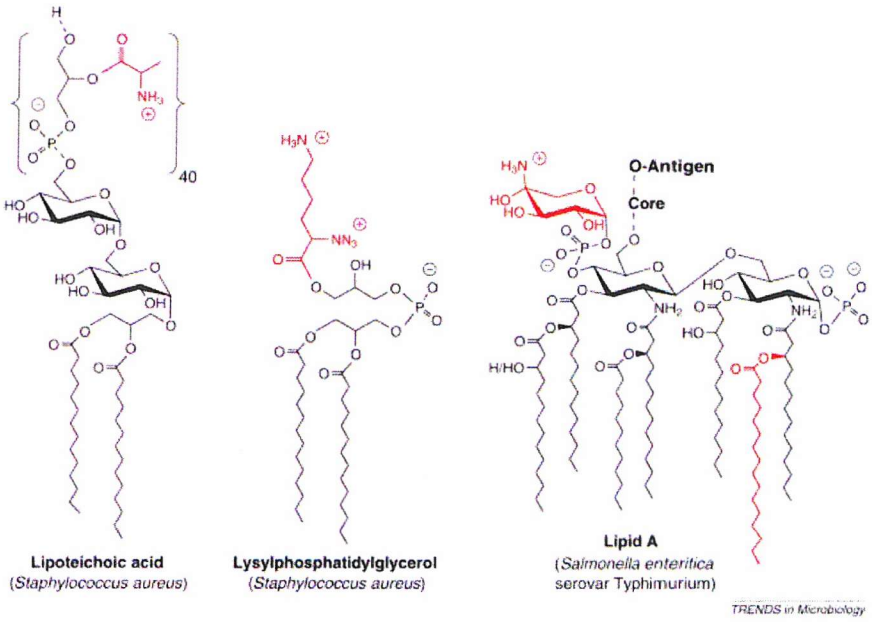


**Figure 3.2** **Structural modifications made to reduce the charge of the bacterial cell envelope**

Modification of the bacterial cell envelope components involved in cationic antimicrobial peptide (CAMP) resistance. The anionic cell envelope components (black) and cationic substituents (red) involved in CAMP resistance of *Staphylococcus aureus* and *Salmonella enterica* are shown taken from (82) with permission.

A reduction in negative charge of the cell wall and cell membranes of bacteria has been shown to play a key role in reducing their susceptibility to CAMPs (83). This strategy should also apply to reducing their susceptibility to the highly cationic gIIA $sPLA_2$ and very recent work on this subject is discussed later, section 3.3.

As discussed previously it seems likely that it is the high charge on the gIIA enzyme that is responsible for the enzyme's ability to penetrate the bacterial cell wall and gain access to the underlying cell membrane where hydrolysis can take place. Similar to bacteria's defensive mechanisms of charge reduction limiting the effectiveness of CAMPs, a reduction in the overall charge on the hgIIA enzymes surface would it might be predicted be accompanied by reduction in cell membrane hydrolysis and consequently its antibacterial potency.

A wide range of charge reversal mutants of recombinant human group IIA $sPLA_2$ had been produced to investigate heparin (84) and anionic vesicle binding (41) and these were available to investigate the charge requirements of its bactericidal activity. These mutants, contained a variety of lysine and arginine residues mutated to aspartate or glutamate leading to a net loss in positive charge of 2 per mutation, see figure 3.3.

This series of mutants were also used to demonstrate whether it was the overall charge on the protein (77) or specific residues or groups of residues (54;85) that enabled it to cross the highly anionic bacterial peptidoglycan layer and thereby gain access to the underlying cell membrane where hydrolysis can take place.

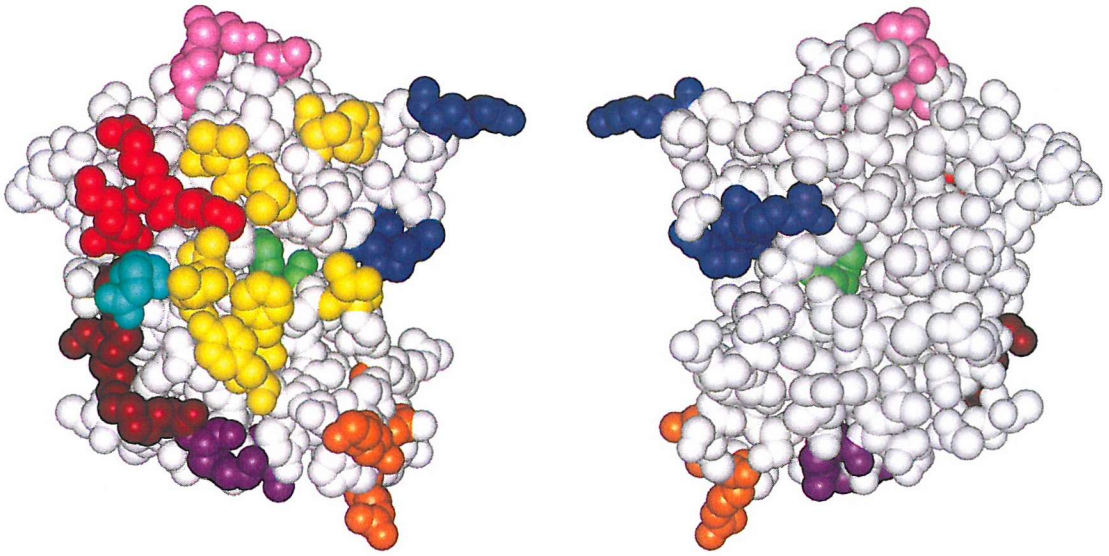

**Figure 3.3** **Space filling representation of hgIIA $sPLA_2$ showing position of cationic residues mutated to anionic**

Cationic residues; red – H6/R7/K10, rust – K16/K110/K115, orange – R34/K124/R127, pink – K74/K87/R92, purple – K38/K116, blue – K53/R54/R58. Anionic residue; cyan – E17, hydrophobic residues; yellow – L2, V3, A19, L20, F24, V31, F70, and Y119 and catalytic site histidine residue: green – H48. The image on the left is viewed with the interfacial binding surface pointing towards the viewer and on the right the opposite face with the enzyme rotated about the vertical axis by 180°.

*Micrococcus luteus* a Gram-positive bacterial strain was used initially for these studies due to their susceptibility to cell wall disruption by lysozyme, which hydrolyses the β1,4 glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid. Lysozyme is an enzyme secreted from a variety of cells in conjunction with hgIIA $sPLA_2$ e.g. macrophages and platelets it is also found in high concentrations in human tears (see section 1.5.2 and (58)). Lysozyme treatment allowed the peptidoglycan layer to be permeabilised so that the native and charge reversal mutants could be assayed directly against the cell membrane as a control for those carried out against untreated cells (intact peptidoglycan layer). Lysozyme treatment provided a relatively non-disruptive method for permeabilising the cell wall while maintaining the integrity of the cell. These studies were extended to include *Staphylococcus aureus* and *Listeria innocua* Gram-positive bacteria to see if any findings were representative of Gram-positive bacteria generally and finally to *Escherichia coli* Gram-negative bacteria which were pre-treated with polymyxin B sulphate an outer membrane permeabilising agent.

## 3.2 Results

### 3.2.1 Comparison of bacterial membrane hydrolysis by various $sPLA_2$s

It has been shown previously that human group IIA $sPLA_2$ is able to hydrolyse suspensions of *M.luteus* in contrast to the enzymes from porcine pancreas (group IB) and *Naja naja* venom (group IA) which showed negligible activity (86). This contrasts markedly with the effect seen on mammalian cells where the opposite can be seen, see figure 3.4 taken from (87) with permission from A.G. Buckland. The investigation of the highly variable ability of different $sPLA_2$s to hydrolyse suspensions of *M.luteus* was extended to encompass a number of other $sPLA_2$s the human group IB, IIE, IIF, V and X and the honeybee venom group III enzyme.

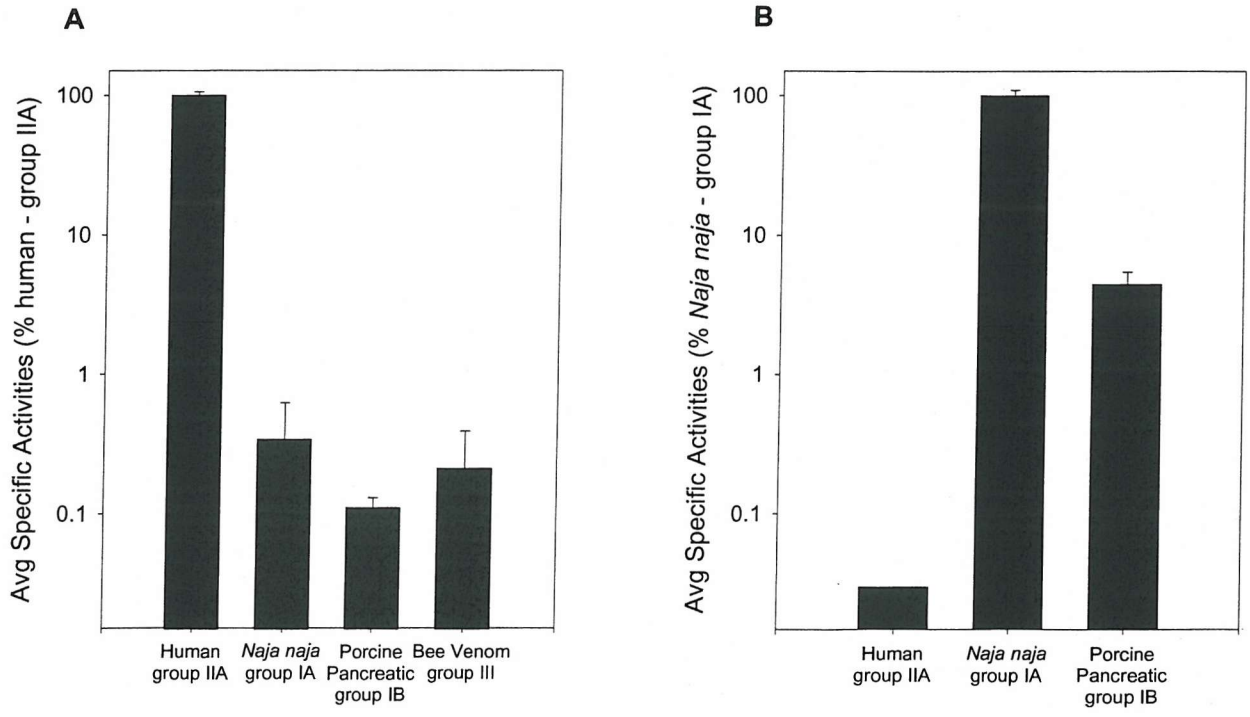


**Figure 3.4** **Initial rates of hydrolysis of *M.luteus* bacterial (A) and L929 fibroblasts eukaryotic (B) cell membranes**

Taken from (87). Average specific activities are plotted on a log scale due to the dramatic differences that are observed. Data shown are means ± S.D. (n=3).

Suspensions of *M.luteus* were mixed with FABP and DAUDA so that fatty acid release resulting from phospholipid hydrolysis could be monitored in real time, various $sPLA_2$s were added to the suspension with or without the prior addition of lysozyme to permeabilise the cell wall. The results, figure 3.5, show that apart from the human group IIA enzyme only the human group V enzyme shows any significant activity with the *M.luteus* suspensions. The group V enzyme has about 15% of the activity of the group IIA enzyme whereas all the other enzymes tested expressed 1-2% of the activity of this enzyme. In the presence of lysozyme all the $sPLA_2$s excluding IIA show improved rates of hydrolysis, consistent with the IIA having relatively unhindered access through the cell wall and the other $PLA_2$s possessing a reduced ability to cross this barrier. Excluding the group III enzyme, the enzymes presented here all have essentially identical sizes and in the case of the group IIE and F predicted structures.

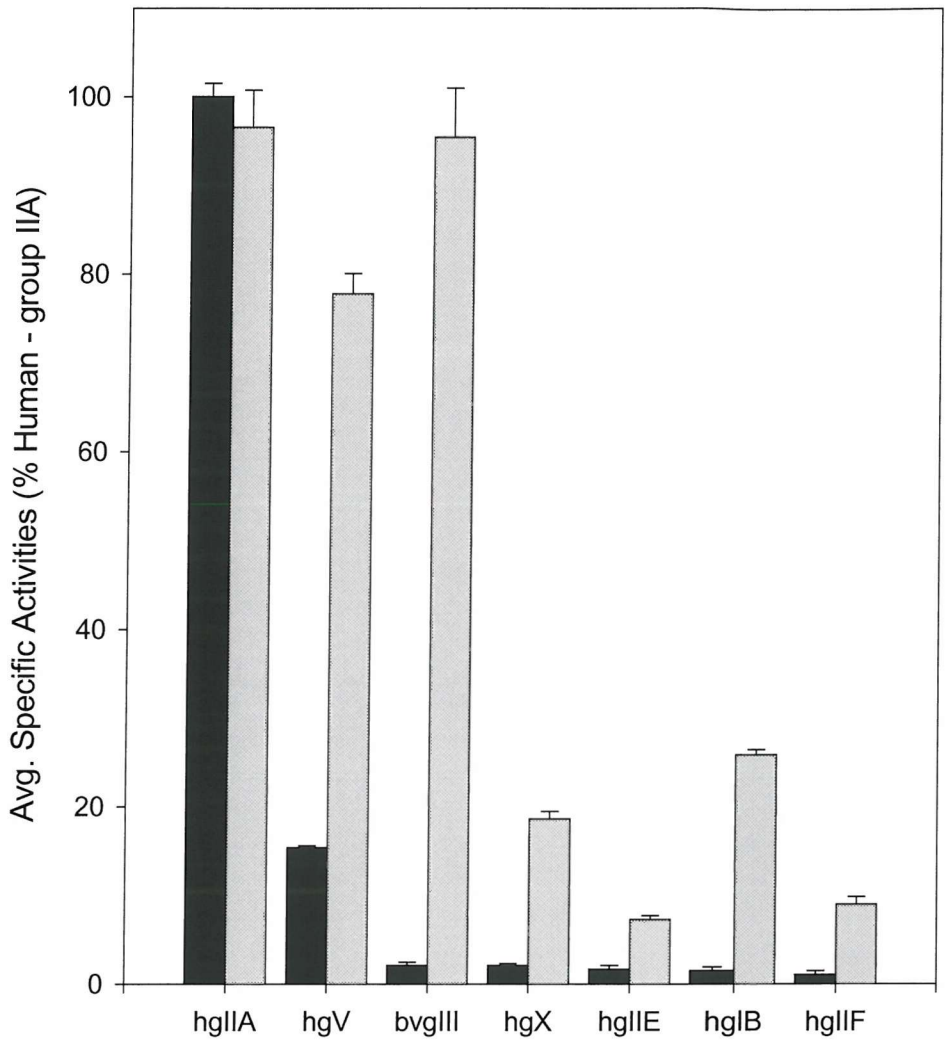


**Figure 3.5** **Activity of various $sPLA_2$s against *M.luteus* suspensions and the effect of lysozyme**

Specific activities were determined for human group IIA (hgIIA), hgV, hgX, hgIIE, hgIB, hgIIF and bee venom group III (bvgIII) $sPLA_2$s (200ng) against intact (black bars) *M.luteus* ($1x10^7$ cells/ml) and chicken egg white lysozyme (20µg) treated cells (grey bars) using a continuous fluorescence displacement assay described in (75). The absolute specific activity value for hgIIA against intact bacteria was 5.3 ± 0.1 nmol/min/µg. Data shown are means ± S.D. (n=3).

An explanation for the difference in permeability of these otherwise similar enzymes is that the IIA enzyme with its highly cationic nature is better able to

penetrate the highly anionic bacterial cell wall (86). The group V enzyme in contrast, although cationic with a net charge of +10 compared to +19 for the group IIA enzyme, only manages relatively low levels of cell wall penetration and hence cell membrane hydrolysis. The remaining enzymes are essentially inactive under the conditions tested, the human group X enzyme is anionic with a tabulated net charge of –3, the IIF –6 and IB a charge of +5 (17). The bee venom group III enzyme (although structurally different to the human $sPLA_2$s tested here) and human group IIE (very recently shown to express little activity on PG and PC interfaces (108)), have a net charge of +9 and these too are essentially inactive on *M.luteus*. The results presented here, with only the group V enzyme (with an overall positive charge of +10) showing significant activity on the *M.luteus* cell suspensions, suggest that there is a correlation between the cationic nature of the $sPLA_2$ and its ability to penetrate the cell wall of *M.luteus*. Cationic enzymes (IIA, V and bee venom III) all expressed high activity on the exposed anionic cell membrane, presumably reflecting the high affinity of these enzymes for the interface.

### 3.2.2 Decreasing positive charge leads to lower levels of hydrolysis of *M.luteus*

The results shown above, figure 3.5, highlight the possible importance of the highly cationic nature of hgIIA $sPLA_2$ in allowing the enzyme to penetrate the anionic bacterial cell wall and thereby gain access to the underlying cell membrane where hydrolysis can occur. In order to demonstrate the importance of the IIA's cationic nature for cell wall penetration a large number of charge reversal mutants (41;84) were assayed against intact *M.luteus* cell suspensions. A series of residues were chosen for mutagenesis that would not only reduce the overall positive charge on the protein but would also identify if any particular regions of positive charge were important rather than the global charge on the proteins surface.

For simplicity the N1A mutant of hgIIA $sPLA_2$ will be referred to as native or wild-type and the charge reversal mutants will be referred to as the R7E/K10E

mutant etc as all these mutants carry the original N1A mutation needed for efficient expression in *E.coli.*

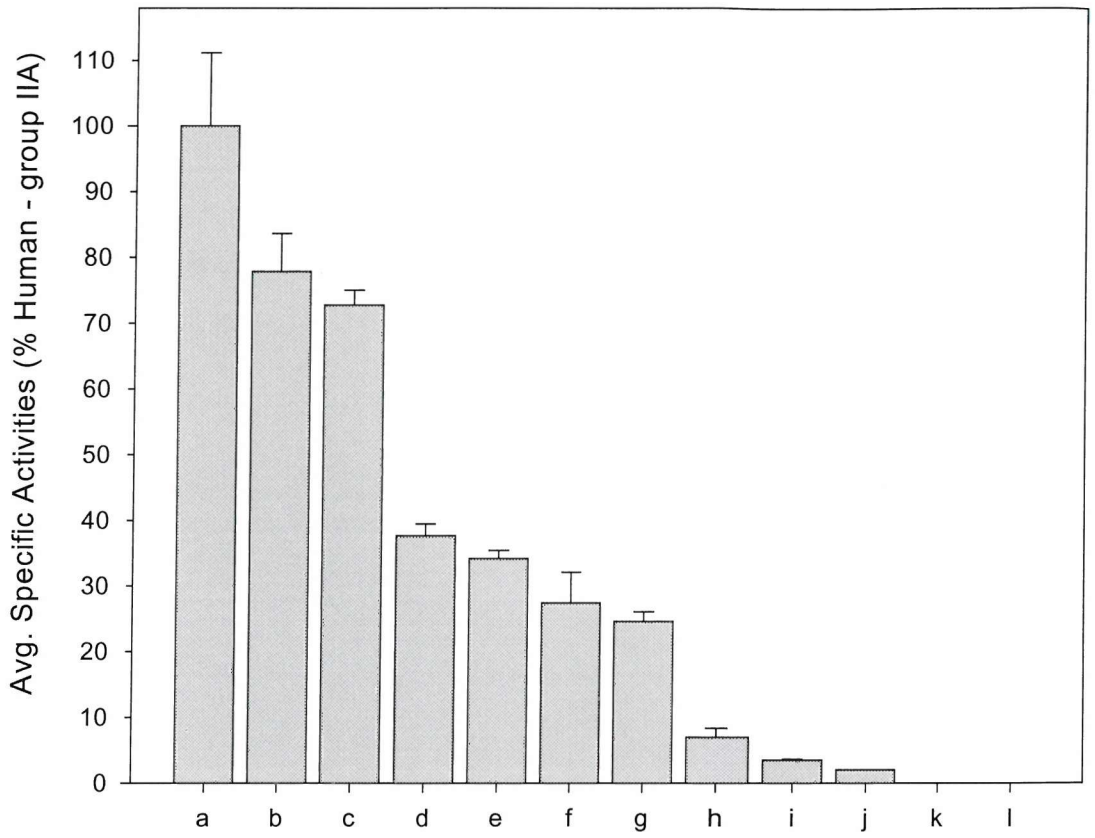


**Figure 3.6** **Ability of native and charge reversal mutants of hgIIA $sPLA_2$ to hydrolyse *M.luteus* cell membranes**

Specific activities were determined for native (a) and charge reversal mutants R7E/K10E (b), K110E/K115E (c), K38E/K116E (d),K10E/K16E (e), K124E/R127D (f), K53E/R54E/R58E (g),K74E/K87E/R92E (h), R7E/K10E/K16E (i), K38E/K124E/R127D (j),K53E/R54E/R58E/K124E/R127D (k), and R7E/K10E/K16E/K124E/R127D (l) of hgIIA $sPLA_2$ against *M.luteus* ($1x10^7$ cells/ml) in HBSS supplemented with 1mM $Ca^{++}$ using a continuous fluorescence displacement assay described in (75). The absolute specific activity value for native hgIIA (a) was 6.6 ± 0.7 nmol/min/µg. Data shown are means ± S.D. (n=3).

Suspensions of *M.luteus* were assayed with the various charge reversal mutants and the rate of $sPLA_2$ activity as measured by fatty acid release was normalised to that seen with the wild type enzyme. Figure 3.6 shows that there

is a direct correlation between the loss in positive charge associated with the differing mutations and the loss in the protein's ability to hydrolyse the bacterial membranes. This loss in activity appears to be non-specific for residues or groups of residues but rather a reflection of the loss in the overall positive charge on the protein surface, as the different mutants contained different clusters of positive charge on the proteins surface (41;84).

### 3.2.3 Charge reversal mutants show equivalent or enhanced activity on DOPG SUVs

The dramatic effect of loss of positive charge on enzyme activity seen in figure 3.6 could be simply due to loss of enzyme activity as a result of mutagenesis. Therefore as a control for the *M.luteus* data it was decided to use DOPG SUVs. It had previously been shown elsewhere that the charge reversal mutants had demonstrated similar activities to wild type using a novel polymerised mixed liposome assay but such an assay has limitations in reflecting the *in vivo* situation (84). In fact, the specific activity of the various mutants varied quite considerably when assayed using DOPG as seen in figure 3.7. In addition to this variance they predominantly showed enhanced activity when compared to the native enzyme, in complete contrast to what may have been expected. Previous studies would have predicted that the native hgIIA $sPLA_2$ would be more active on such anionic vesicles due to its considerably higher binding affinity $K_d$ ~$10^{-10}$M when compared to the charge reversal mutants, for example R7E/K10E/K16E at $K_d$ ~$10^{-7}$M (41).

At this time there is no an explanation for the considerably enhanced catalytic activity seen with DOPG as substrate for some of the charge reversal mutants, particularly those involving mutations at the N-terminal. These *major differences were not seen previously using the polymerised liposome assay* (41). The possible molecular basis for this enhanced activity is discussed later in this chapter (see section 3.2.7).

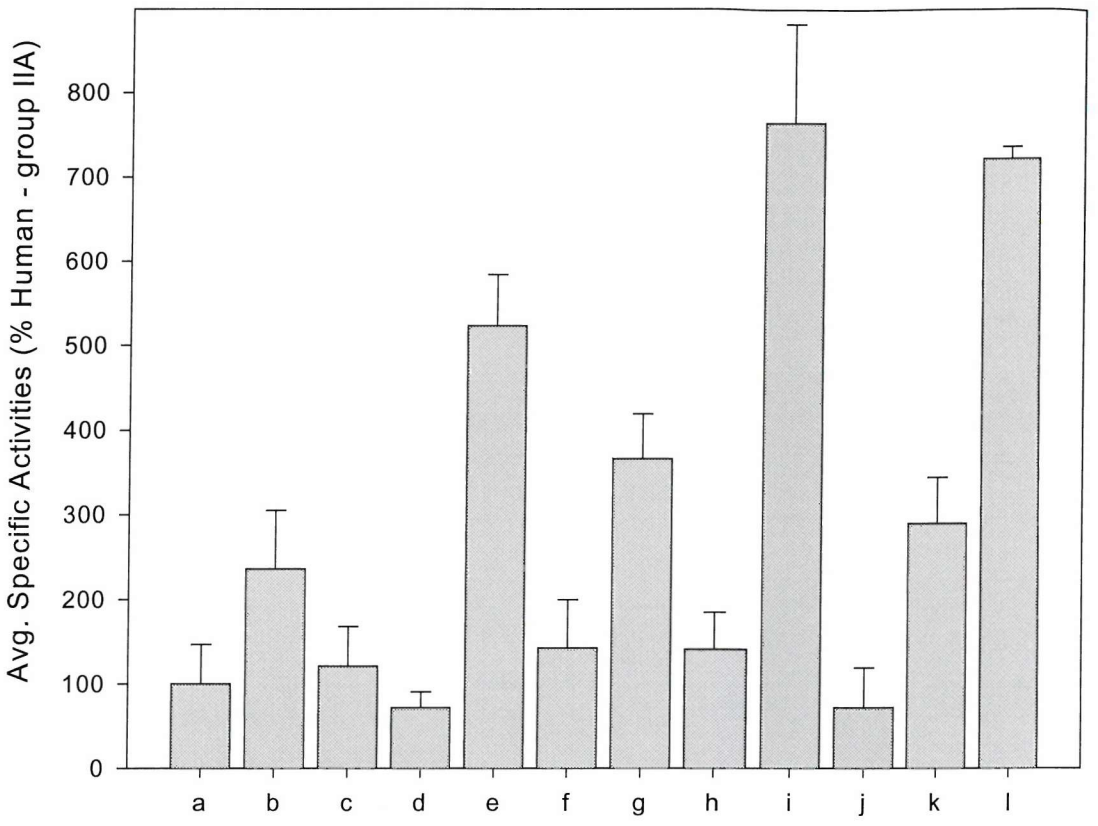


**Figure 3.7** **Activities of native and charge reversal mutants of hgIIA $sPLA_2$ against DOPG SUVs**

Specific activities were determined for native (a) and charge reversal mutants R7E/K10E (b), K110E/K115E (c), K38E/K116E (d),K10E/K16E (e), K124E/R127D (f), K53E/R54E/R58E (g),K74E/K87E/R92E (h), R7E/K10E/K16E (i), K38E/K124E/R127D (j),K53E/R54E/R58E/K124E/R127D (k), and R7E/K10E/K16E/K124E/R127D (l) of hgIIA $sPLA_2$ against DOPG SUVs (63nmols $ml^{-1}$) in 0.1M Tris.HCl and 0.1M NaCl supplemented with 1mM $Ca^{++}$ using a continuous fluorescence displacement assay described in (75). The absolute specific activity value for native hgIIA (a) was 80 ± 37 nmol/min/µg. Data shown are means ± S.D. (n=3).

### 3.2.4 Lysozyme restores hydrolytic activity to charge reversal mutants on *M.luteus*

In order to ascertain whether the lower activity of the mutants against cells was indeed due to a reduced ability to penetrate the anionic cell wall the cells were pre-treated with lysozyme. This enzyme hydrolyses the cross-links between strands of peptidoglycan, the highly anionic and cross-linked material within the cell wall, and we have already shown that lysozyme treated cells become more permeable to other less cationic $sPLA_2$s.

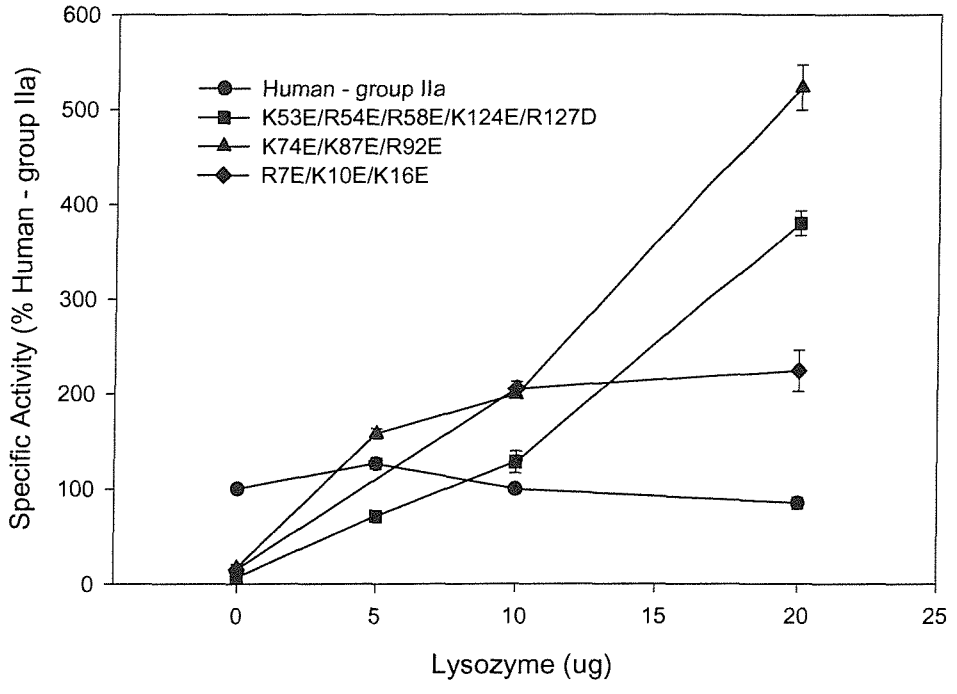


**Figure 3.8** **Effect of lysozyme pre-treatment on the ability of native and charge reversal mutants of human group IIA $sPLA_2$ to hydrolyse cell suspensions of *M.luteus***

Cells suspensions of *M.luteus* ($1x10^7$ cells/ml) in HBSS supplemented with 1mM $Ca^{++}$ were incubated with varying amounts of lysozyme (from chicken egg white) for 2 minutes prior to the addition of native (●) and charge reversal mutants K53E/R54E/R58E/K124E/R127D (■), K74E/K87E/R92E (▲) and R7E/K10E/K16E (♦) of human group IIA $sPLA_2$. Specific activities were determined using a continuous displacement assay described in (75). The absolute specific activity value for native hgIIA (●) against intact bacteria was 5.5 ± 0.4 nmol/min/µg. Data shown are means ± S.D. (n=3).

As can be seen from figure 3.8, which compares the native hgIIA $sPLA_2$ with K53E/R54E/R58E/K124E/R127D, K74E/K87E/R92E and R7E/K10E/K16E mutants, the addition of lysozyme had no great effect on the native enzymes ability to hydrolyse phospholipid. However in marked contrast a dramatic increase in the activity of the three charge reversal mutants is demonstrated. This result suggests that as the bacterial cell wall is degraded the mutant enzymes, with their lower positive charge, are able to gain access to the cell membrane and exhibit higher activity. Moreover, the expressed activity of the mutants with lysozyme treated cells was considerably greater than for the wild type enzyme. Thus it would appear that the unusual enhanced activity demonstrated by charge reversal using DOPG SUVs (figure 3.7) is also demonstrated using bacterial cell membranes.

Overall, this result using lysozyme supports the contention that the highly basic nature of $sPLA_2$ is required for it to exhibit its potent Gram-positive antibacterial response as it is this charge that permits it to penetrate and cross the anionic cell wall of these bacteria.

### 3.2.5 The role of positive charge in the hydrolysis of *S.aureus* and *L.innocua* cell membranes

The importance of charge in the ability of hgIIA to hydrolyse *M.luteus* membranes had been established and the study was extended to encompass *Staphylococcus* and *Listeria* species to show whether this was a general Gram-positive phenomenon. Previous studies have shown that both *Staphylococcus* and *Listeria* are sensitive to hgIIA $sPLA_2$ (58). In order to preserve limited stocks of the charge reversal mutants a two-site mutant (K124E/R127D) and five-site mutant (K53E/R54E/R58E/K124E/R127D) were used.

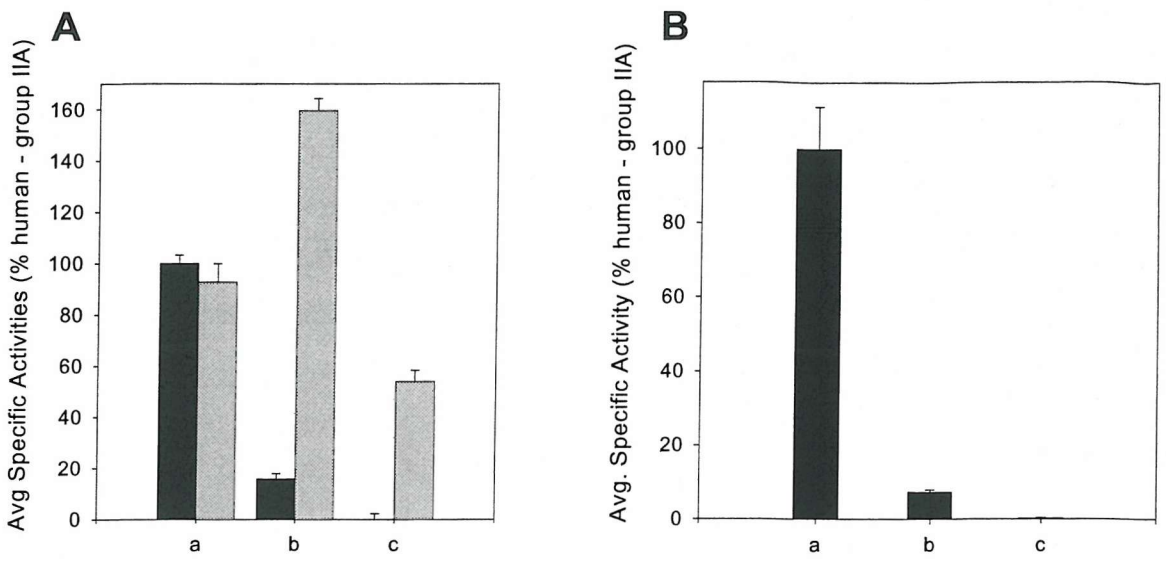


**Figure 3.9** **The ability of native and charge reversal mutants of human group IIA $sPLA_2$ to hydrolyse *S.aureus* (A) and *L.innocua* (B) membranes**

Specific activities were determined for native(a) and charge reversal mutants K124E/R127D (b), and K53E/R54E/R58E/K124E/R127D (c) of hgIIA $sPLA_2$ (150ng) against intact (black bars) and lysostaphin (430ng) pre-treated (grey bars) *S.aureus* (A) ($2 \times 10^7$ cells/ml) and intact *L.innocua* (B) ($2 \times 10^8$ cells/ml) membranes using a continuous fluorescence displacement assay described in (75). The absolute specific activity values for native hgIIA (a) against intact bacteria were 6.7 ± 0.2 (**A**) and 9.7 ± 1.1 (**B**) nmol/min/μg. Data shown are means ± S.D. (n=3).

Suspensions of *S.aureus* and *L.innocua* were treated with $sPLA_2$ and fatty acid release was monitored using the DAUDA displacement assay. The results in figure 3.9 show that the two site mutant demonstrates a partial loss in ability to hydrolyse both *S.aureus* (A) and *L.innocua* (B) cell membranes and that the five site shows negligible activity against the cell suspensions, confirming the pattern seen with *M.luteus* (figure 3.9). It is not possible to permeabilise the cell wall of either *S.aureus* or *L.innocua* using lysozyme (data not shown) however it is possible to selectively permeabilise the cell wall of *S.aureus* using lysostaphin. Lysostaphin is a cell wall protease from *Staphylococcus staphylolyticus* that is composed of a hexosaminidase, an amidase and the major component an endopeptidase which cleaves polylgycine cross-links in the cellular wall to liberate N-terminal glycine and alanine from *S.aureus* (88). The results (figure 3.9A) clearly show that prior

treatment of suspensions of *S.aureus* with lysostaphin allowed full expression of group IIA mutant activity due to cell wall hydrolysis.

### 3.2.6 Effect of salt concentration on hgIIA hydrolysis of *M.luteus* cell membranes

The results described for *M.luteus* (figures 3.6 and 3.8) and *S.aureus* and *L.innocua* (figure 3.9) highlight the importance of electrostatic interactions between the cationic enzyme surface and the anionic cell wall in allowing the enzyme access to the underlying cell membrane. In support of these findings assays were carried out with varying salt concentrations and the effects shown below.

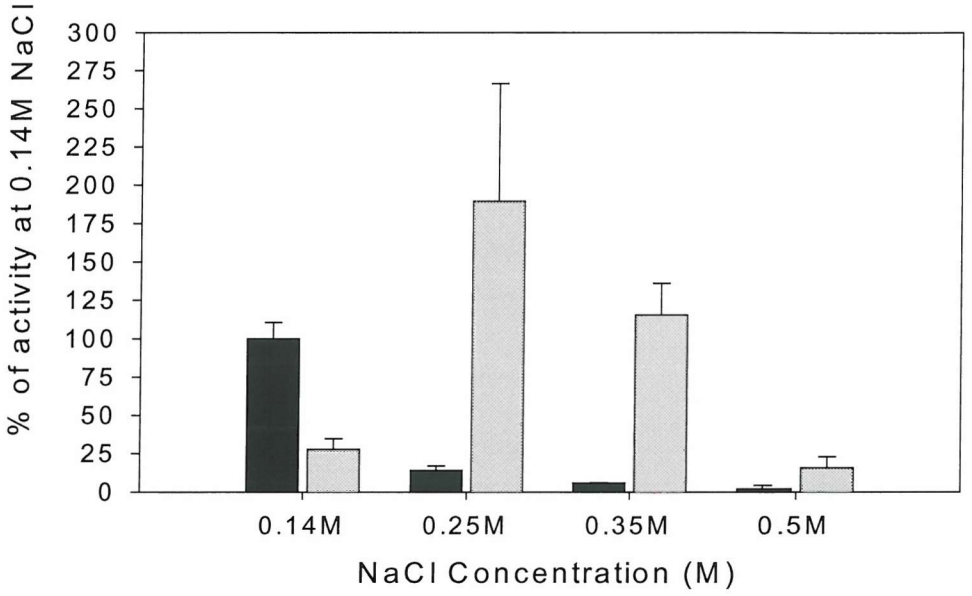


**Figure 3.10** **Effect of salt concentration on the ability of hgIIA $sPLA_2$ to hydrolyse suspensions of *M.luteus***

Specific activities were determined for hgIIA $sPLA_2$ against *M.luteus* (1 x $10^7$ cells/ml), using a fluorescence displacement assay described in (75). Assays were performed in HBSS containing 0.14M NaCl and supplemented with additional salt to 0.5M as indicated (black bars). For lysozyme pre-treated cells (grey bars) the initial incubation was performed in 0.14M salt and the salt increased prior to addition of $sPLA_2$. The absolute specific activity value for hgIIA against intact bacteria in 0.14M NaCl was 2.3 ± 0.2 nmol/min/µg. Data shown are means ± S.D. (n=3).

It can be seen from figure 3.10 that as electrostatic interactions between the protein and the bacterial cell wall are disrupted by increasing salt concentration the enzymes ability to hydrolyse the intact cell suspensions was inhibited. In order to show that under these salt conditions the enzyme was still active on the cell membranes themselves the cells were pre-treated with lysozyme and again assayed with enzyme under varying salt conditions. The results show that once the cell wall is perturbed the enzyme is able to demonstrate quite high activity on the exposed cell membrane, similarly to those patterns seen with the charge reversal mutants (figure 3.8). A biphasic response is seen with increasing salt concentration for the lysozyme treated cells. Initially the activity is enhanced followed by decreasing activity at higher salt concentrations. Interfacial binding is a complex process involving both electrostatic and non-polar interactions, both of which will be affected by salt concentration.

These results would suggest that hgIIA $sPLA_2$ pays some form of "catalytic price" for its ability to pass through the outer cell wall of Gram-positive bacteria. The enzyme it might be suggested is binding too tightly to the membrane in a relatively catalytically unproductive manner, and this low activity state is relieved by decreasing charge (figure 3.8) or by increased salt concentration (figure 3.10). Figure 3.10 demonstrates that the high surface charge of hgIIA $sPLA_2$ is required for its passage across the outer cell wall of the bacteria and suggests that this charge reduces its ability to hydrolyse the cell membranes by inhibiting interfacial activation in some way. It should be noted that without this charge it would not be able to gain access to the underlying membrane at all.

### 3.2.7 Native hgIIA $sPLA_2$ binds more tightly to DOPG SUVs than charge reversal mutants

The surprising data shown in figure 3.7 must have a molecular explanation. Because the binding of the IIA enzyme to anionic vesicles must be dominated by electrostatic interactions it was anticipated that charge reversal mutants

would bind relatively less tightly to DOPG vesicles and hence their activity would be more sensitive to salt concentration. In the case of the 3-site mutant this is indeed the case. When activities are compared under normal conditions and in the presence of 0.5M NaCl, the mutant is clearly less active than under normal assay conditions. In contrast, the activity of the native enzyme is minimally affected by the high salt concentration (see figure 3.11).

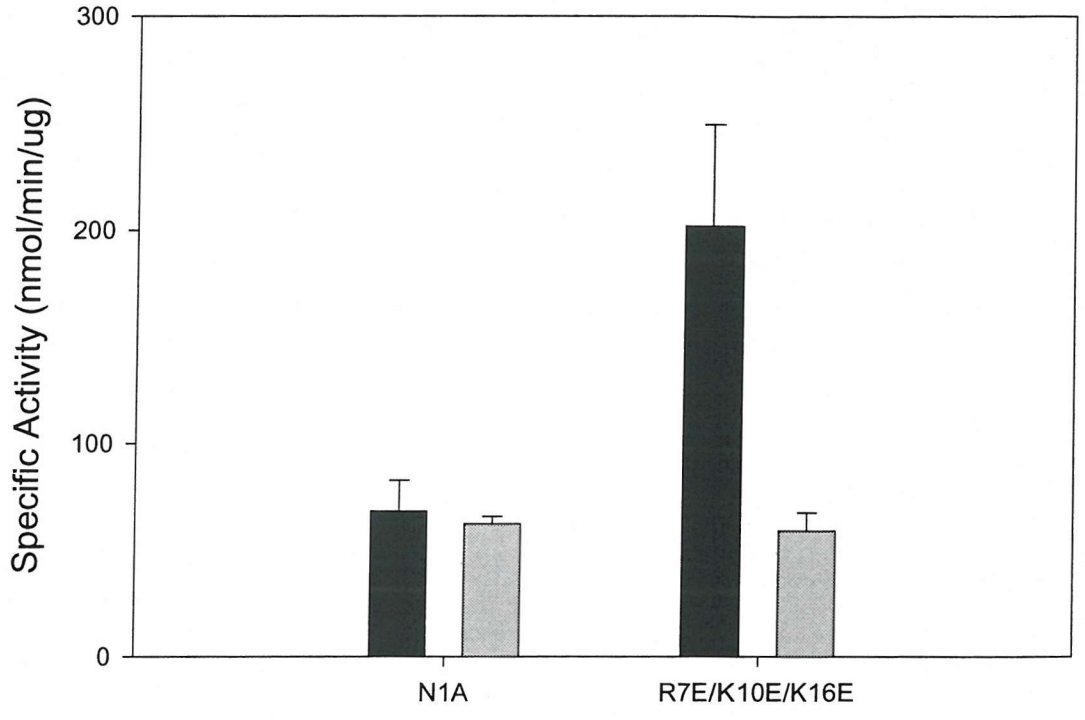


**Figure 3.11** **Effect of salt concentration on the activities of native and R7E/K10E/K16E charge reversal mutant of hgIIA sPLA$_2$ against DOPG SUVs**

Specific activities were determined for native and a charge reversal mutant of hgIIA sPLA$_2$ against DOPG SUVs (63nmols/ml) in 0.1M Tris.HCl and 0.1M NaCl (black bars) and supplemented with additional salt to 0.5M (grey bars). Data shown are means $\pm$ S.D. (n=3).

This suggests that the enhanced activity seen with the mutants under normal assay conditions, and also when mutants are added to permeabilised cells is due to specific electrostatic interactions with the interface that cannot be seen

with the native enzyme. A possible explanation is that, due to multiple electrostatic interactions, the wild type enzyme takes up an unfavourable conformation at the interface relative to the mutants leading to unfavourable rates of catalysis. It should be noted that only those charge reversal mutants involving the interfacial binding surface show enhanced activity with DOPG SUVs. If the conformation of the protein at the interfacial surface is critical for optimum catalysis, then the physical nature (quality) of the interface may also affect the relative activity of wild type and charge reversal mutants.

### 3.2.8 Human group IIA $sPLA_2$ is affected by the quality of the interface

Interface curvature will have a major effect on the physical nature of the interface. As mentioned previously the DOPG results run in marked contrast with previous work (41). SUVs have a radius of approximately 25-50nm much smaller than the LUVs at approximately 100-400nm with which the previous assays had been performed (41). So to test whether the relative activities seen with SUVs was due to increased headgroup exposure or some other aspect of increased curvature of the interface, it was decided to test the enzymes against larger MLVs and then to sonicate these to produce SUVs.

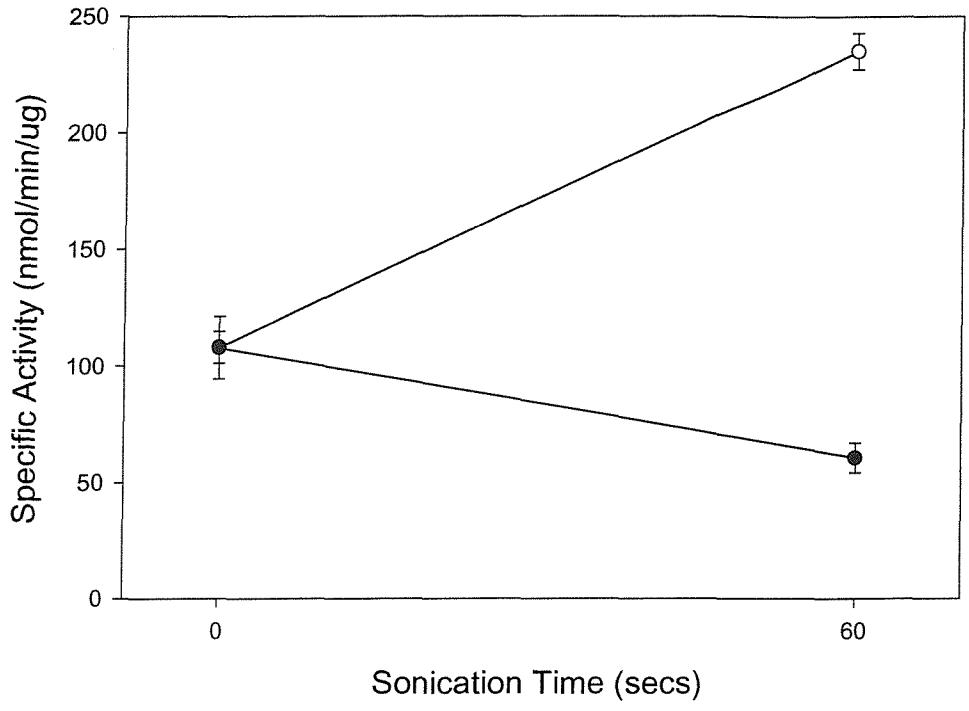


**Figure 3.12 Effect of sonicating DOPG MLVs on the activities of native and R7E/K10E/K16E charge reversal mutant of hgIIA $sPLA_2$**

Specific activities were determined for native (•) and charge reversal mutant R7E/K10E/K16E (○) of hgIIA $sPLA_2$ against DOPG MLVs (63nmols $ml^{-1}$) in 0.1M Tris.HCl and 0.1M NaCl and MLVs sonicated for 60 seconds under the same conditions. Data shown are means ± S.D. (n=3).

As can be seen from figure 3.12 both the native hgIIA $sPLA_2$ enzyme and R7E/K10E/K16E display similar levels of activity against MLVs. The native enzyme showed increased and R7E/K10E/K16E reduced activity towards DOPG when present as MLVs that have a flatter interface (than SUVs). On sonicating the MLVs there is a marked change in activities with a decrease of approximately 40% in the native hgIIA $sPLA_2$, and an increase of approximately 100% in R7E/K10E/K16E bringing them back to similar relative activities as seen previously with SUVs (figure 3.7).

### 3.2.9 *In vitro* antibacterial properties of various human $sPLA_2$s and charge reversal mutants of human group IIA $sPLA_2$

The results seen in figure 3.5 highlight the ability of various $sPLA_2$s to catalyse cell membrane hydrolysis of *M.luteus*. In order to confirm that phospholipid hydrolysis was paralleled by antibacterial activity, *M.luteus* cells suspensions were treated with 1.87μg/ml of enzyme for up to 2 hours and the bacterial viability assessed. As can be seen from Figure 3.13A, the group IIA enzyme was clearly more effective than the group V whilst under these conditions there was minimal loss of viability on treatment with the group IB and X enzymes.

The loss of hydrolysing ability seen with the charge reversal mutants on *M.luteus* suspensions in Figure 3.6 was paralleled by a loss in antibacterial activity as seen in Figure 3.13B. The 2-site mutant R7E/K10E retained significant antibacterial activity whereas no significant activity was seen with the 5-site mutant K53E/R54E/R58E/K124E/R127D with the 3-site K74E/K87E/R92E positioned somewhere between the two.

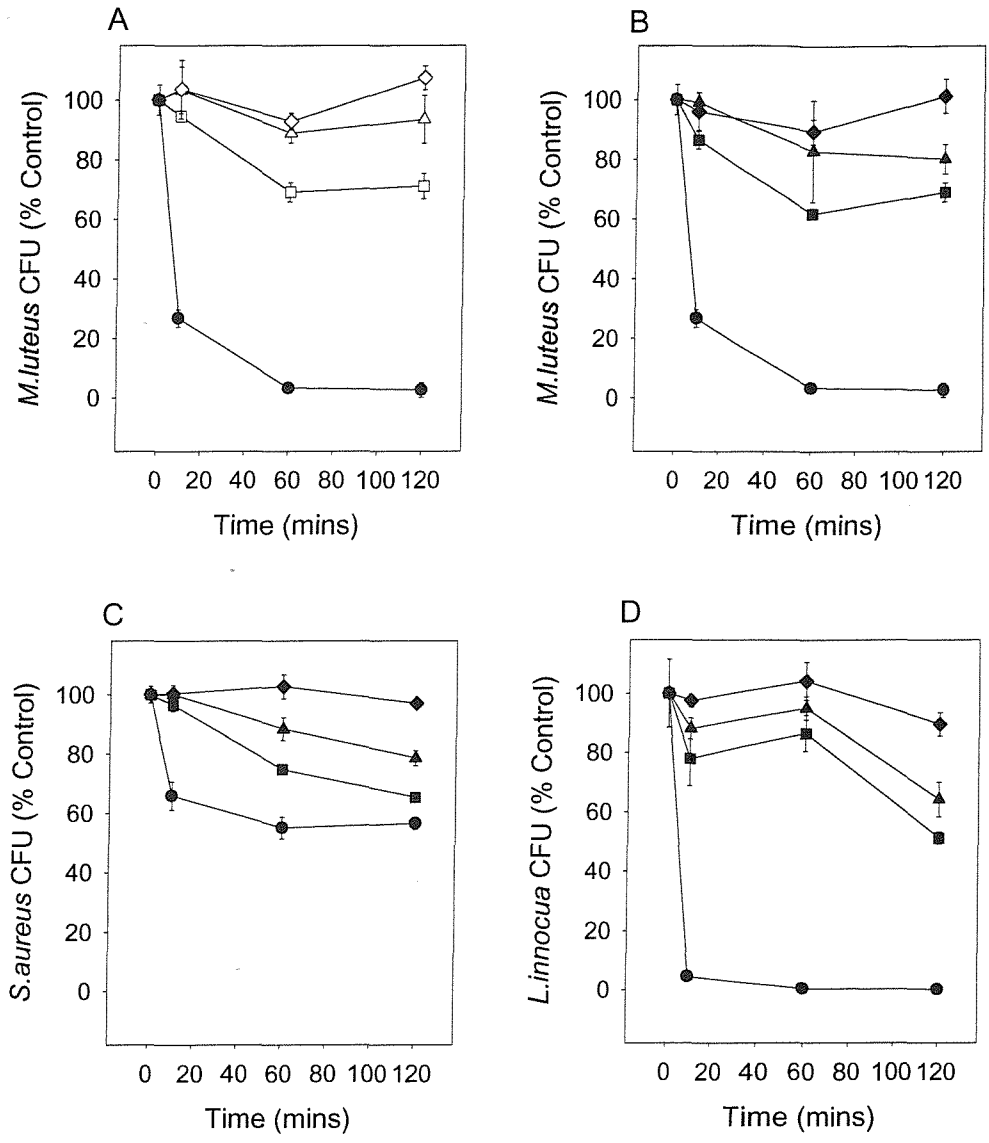


**Figure 3.13** ***In vitro* bactericidal activities of human sPLA$_2$s**

Bacterial viability was assessed by measuring colony-forming ability as described in "Materials and Methods". (A) *M.luteus* suspensions (1 x $10^7$ cells/ml) after treatment with 1.87µg/ml of human groups IIA (●), V (□), X (Δ) and IB (◇) sPLA$_2$s. (B-D) The effect of native (●) and charge reversal mutants R7E/K10E (■), K74E/K87E/R92E (▲), and K53E/R54E/R58E/K124E/R127D (◆) of human group IIA sPLA$_2$ on (B) *M.luteus* suspensions (1 x $10^7$ cells/ml) after treatment with 1.87µg/ml, (C) *S.aureus* suspensions (2 x $10^7$ cells/ml) after treatment with 20µg/ml and (D) *L.innocua* suspensions (2 x $10^8$ cells/ml) after treatment with 187ng/ml of enzyme. Data shown are means ± S.D. (n=3).

The same trend was seen with cultures of *S.aureus* (Figure 3.13C) and *L.innocua* (Figure 3.13D). It should be note however that *L.innocua* were considerably more sensitive to the IIA enzyme than *S.aureus* while *M.luteus* cultures were intermediate between the two. Concentrations of enzyme required to produce the results shown in Figures 3.13B-D were 1.87, 20 and 0.187 µg/ml respectively and the 100 fold difference in sensitivity to the IIA enzyme between *Listeria* and *Staphylococcus* was consistent with previous data (58) using the $sPLA_2$ from human tears. The antibacterial activities shown were gained in the presence of 50mg/ml BSA, as it was found that the assays were more sensitive in its presence (data not shown) as has been seen previously (89). This observation is entirely consistent with the protein's ability to bind fatty acids and lysophospholipids, the products of cell membrane hydrolysis, and thereby reduce their re-utilisation by the bacteria in culture. It has been shown that *S.aureus* (89) and *M.luteus* (86) bacteria are able to survive extensive phospholipid hydrolysis by hgIIA $sPLA_2$ in the absence of BSA. During $sPLA_2$ treatment it has been shown that *S.aureus* are able to synthesise membrane phospholipids through separate *de novo* and salvage pathways and the susceptibility of the bacteria to $PLA_2$ activity is dependent on the relative rates of phospholipid degradation and synthesis (89). It should be noted that *in vivo* hgIIA $sPLA_2$ would never be present in isolation but in the presence of a wide variety of other factors in the extra-cellular milieu, such as serum albumin and its increased effectiveness in the presence of such factors would be expected.

### 3.2.10 Decreasing positive charge leads to lower levels of hydrolysis of *E.coli* pre-treated with polymyxin B sulphate

Gram-negative bacteria are resistant to the effects of $sPLA_2$s in the absence of outer membrane permeabilising agents such as BPI (see Chapter 1, section 1.5.2) (54;85). BPI was not available but polymyxin B sulphate is a well-characterised antibacterial reagent that perturbs the lipopolysaccharide coat of Gram-negative bacteria such as *E.coli*. Moreover it has previously been reported that polymyxin B facilitates the hydrolysis of cell membranes by

$sPLA_2$ (90). Therefore, it was decided to test whether partial treatment with this permeabilising agent would allow access of the $sPLA_2$ to the underlying cell wall and hence an evaluation of the effect of surface charge on cell wall penetration and subsequent membrane hydrolysis.

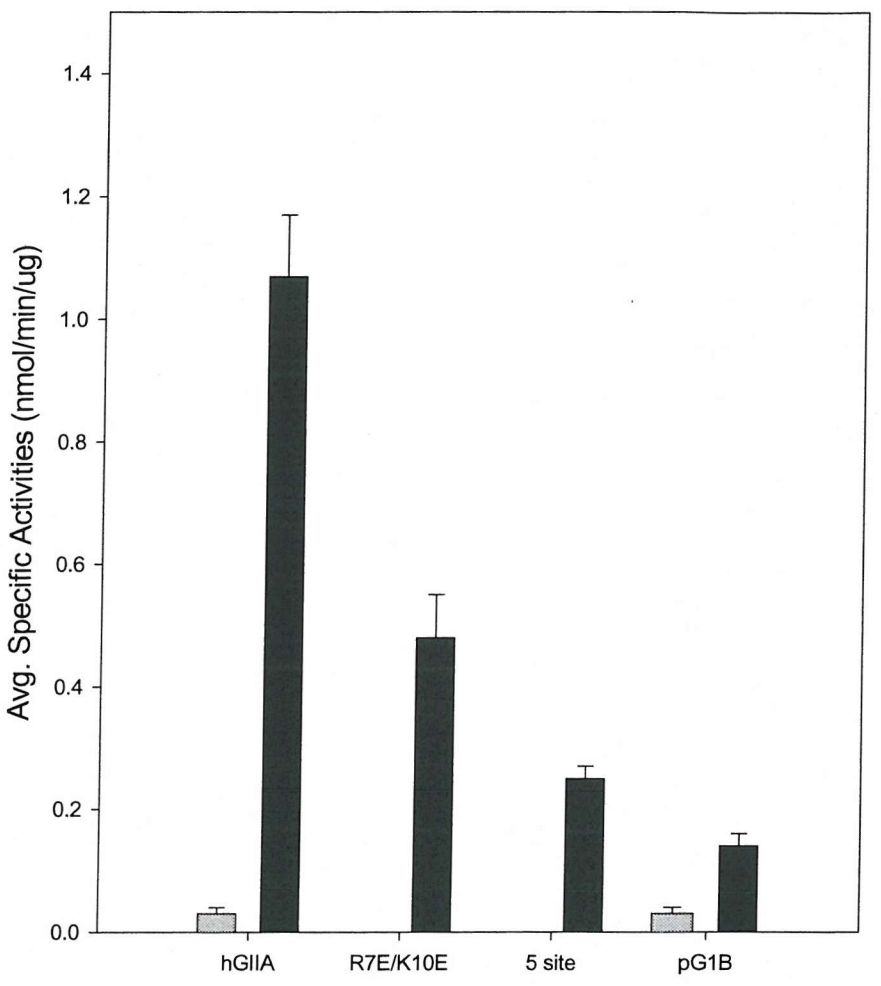


**Figure 3.14** **Ability of $sPLA_2$ to hydrolyse the cell membranes of *E.coli* pre-treated with polymyxin B sulphate**

Specific activities were determined for native and charge reversal mutants R7E/K10E, and K53E/R54E/R58E/K124E/R127D (5 site) of human group IIA $sPLA_2$ together with porcine pancreatic group 1B $sPLA_2$ against *E.coli* ($1x10^7$ cells/ml). The *E.coli* were left untreated (grey bars) or incubated with 0.5ug polymyxin B sulphate for 20 minutes at 37°C (black bars) and assays carried out in HBSS supplemented with 1mM $Ca^{++}$ using a continuous fluorescence displacement assay described in (75). Data shown are means ± S.D. (n=3).

As can be seen from figure 3.14 neither hgIIA or porcine pancreatic group IB shows any significant rate of hydrolysis on untreated cells as expected (54;85). In the presence of polymyxin B sulphate all the $sPLA_2$s showed enhanced activity and the degree of enhancement was directly correlated to the amount of charge present on that enzyme, reflecting the picture seen with the untreated Gram-positive bacteria. However, there was significant hydrolysis with all the $sPLA_2$s tested and this may reflect the more minimal nature of the cell wall in Gram-negative bacteria (see section 3.1).

### 3.2.11 Lysozyme restores hydrolytic activity to charge reversal mutants on polymyxin B sulphate treated *E.coli*

The results shown earlier regarding the Gram-positive bacteria figures 3.5, 3.8, 3.9 and 3.10 demonstrated that in the presence of the cell wall disrupting enzymes lysozyme and lysostaphin $sPLA_2$s generally showed enhanced activity and there was less difference associated with the charge of the enzyme. In order to test whether in the absence of the peptidoglycan layer of *E.coli* $sPLA_2$s would again express more similar activities, lysozyme treatment was incorporated into the polymyxin B sulphate incubations.

Figure 3.15 demonstrates that enhanced activity is seen with *E.coli* as well as the Gram-positive bacteria investigated earlier. Although as stated before no $sPLA_2$s demonstrate appreciable activity on untreated Gram-negative bacteria. Human group IIA $sPLA_2$ with its high pI again seems most suited to the task given that with partial treatment using the permeabilising agent polymyxin B sulphate and polymyxin B sulphate together with lysozyme it demonstrates the higher activity. It should be noted that these studies with Gram-negative bacteria are at a preliminary stage and will need to be expanded greatly before a clear picture is obtained. However, these initial findings suggest that charge may have a role to play in Gram-negative antibacterial activity by $sPLA_2$s and support the concept that only $sPLA_2$ with a very high pI are able to access the cell membrane, once the polysaccharide coat has been permeabilised. This characteristic is entirely consistent with the

properties of BPI. It was shown that only certain $sPLA_2$s with a high pI were BPI sensitive (85) while more recent studies have established that BPI is able to disrupt the lipopolysaccharide coat (91;92).

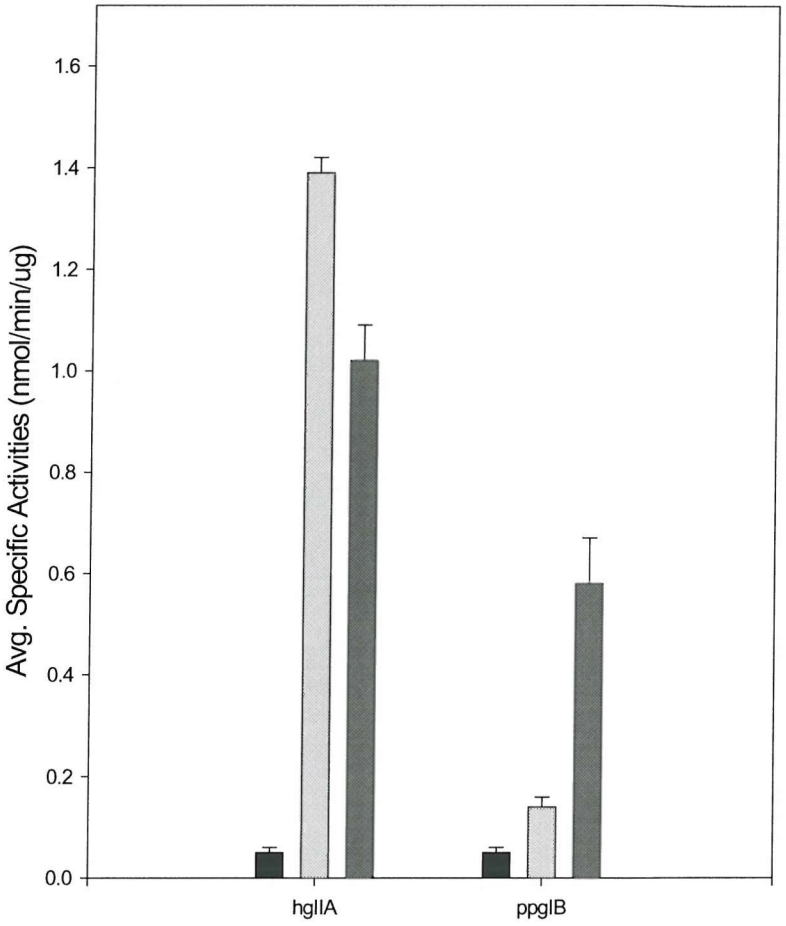


**Figure 3.15** **Effect of lysozyme treatment on the ability of $sPLA_2$ to hydrolyse the cell membranes of *E.coli* pre-treated with polymyxin B sulphate**

Specific activities were determined for human group IIA $sPLA_2$ and porcine pancreatic group 1B $sPLA_2$ against *E.coli* ($1x10^7$ cells/ml). The *E.coli* were left untreated (black bars) or incubated with 0.5ug polymyxin B sulphate for 20 minutes at 37°C (light grey bars) or 0.5ug polymyxin B sulphate for 20 minutes and 20ug lysozyme for 5 minutes (dark grey bars). Assays were carried out in HBSS supplemented with 1mM $Ca^{++}$ using a continuous fluorescence displacement assay described in (75). Data shown are means ± S.D. (n=3).

### 3.3 Discussion

The precise physiological roles of human group IIA $sPLA_2$ remain unclear and the ever-expanding number of $sPLA_2$s discovered with similar size and predicted structure has added to the complexity of the situation (17). The fact that these enzymes are so very similar in structure and have only modest differences in active site substrate specificity focuses attention on the interfacial and other surface properties of these enzymes. As interfacial enzymes, $sPLA_2$ need to be able to identify the correct target membrane, bind to it and subsequently access the phospholipid substrate in a productive manner. Arguably, which membranes and consequently what substrates are available to the different $sPLA_2$ might be expected to depend on differences in the surface characteristics of the proteins, the membranes to which they are exposed and other factors of the extra-cellular environment in which they operate.

The surface properties of the human group IIA enzyme are dominated by two characteristics; it is extremely cationic in nature and has no interfacial tryptophan residues (12). The enzyme has a marked preference for anionic phospholipid interfaces, characteristic of the outer leaflet of bacterial cells. In marked contrast the enzyme exhibits an almost complete lack of activity on zwitterionic interfaces such as those normally present on the outer leaflet of eukaryotic cell membranes. The inability to hydrolyse such neutral phospholipid aggregates has been proposed to reflect a lack of affinity for such interfaces, due at least in part to an absence of interfacial tryptophans (12;48). Only when the enzyme is applied at levels far in excess of that seen *in vivo,* except perhaps under the most extreme inflammatory conditions (21;46;48;84), is activity evident. Such characteristics make the enzyme particularly well designed for it to have selective antibacterial properties.

The antibacterial potential of the group IIA enzyme has been recognised for some time (reviewed in (77)) and the importance of surface positive charge in allowing penetration of the wall has been discussed (77). Other human

$sPLA_2$s do not have the extreme cationic nature commensurate with this antibacterial function although the human group V enzyme does show considerable cationic character.

In this chapter the potential of the human group IB, IIA, IIE, IIF, V and X enzymes to hydrolyse bacterial cell membranes has been studied. The results clearly demonstrate that the group IIA has enhanced ability to hydrolyse the cell membrane of suspensions of intact Gram-positive bacteria, a characteristic that reflects the ability of the enzyme to penetrate the anionic peptidoglycan cell wall. Once the permeability barrier of the cell wall is removed, then all enzymes expressed more similar activity against the exposed anionic bacterial cell membrane.

The bacterial cell wall is a structurally complex barrier that varies considerably from organism to organism. Therefore the permeability properties to a large molecule such as a $sPLA_2$ will vary accordingly. In the case of *M.luteus*, this has provided a good model system because the IIA enzyme appears to be fully permeable to the cell wall in comparison to other $sPLA_2$s while the cell wall can be made permeable by treatment with lysozyme. Similarly we found that *L.innocua* was very sensitive to the action of the IIA enzyme. It was the most sensitive to hydrolysis of the organisms tested, confirming published data (58). In contrast, other bacteria such as *S.aureus* have a more condensed cell wall and permeability of even the IIA enzyme is restricted and may be sensitive to the physiological state of the organism (89), possibly reflecting the degree of cross-linking.

It is noteworthy that the group V enzyme, which is also a basic protein (tabulated net charge +10), has a modest ability to penetrate the cell wall. The basic bee venom enzyme (tabulated net charge +9) was included in this comparison and also was unable to demonstrate significant ability to hydrolyse *M.luteus* cell suspensions. However, the structure of this enzyme is sufficiently different from group I and group II enzymes to make direct comparisons more difficult. Our understanding of the structure and function of the group IIE, IIF

and X enzymes is at an early stage, however, the data presented would suggest that these enzymes do not have a bactericidal role in the body.

The major role of surface cationic charge in allowing the group IIA enzyme to penetrate the highly anionic cell wall has been confirmed by structural studies involving charge reversal mutagenesis in which surface cationic residues have been turned into anionic residues. It is clear that the loss of cell wall permeability correlated with loss of positive charge while by the time that 5 site mutations had been introduced, giving a net positive charge of 9, this permeability was less than 1% of the wild-type enzyme. This loss of permeability was monitored as reduced access to the underlying cell membrane resulting in corresponding reduced rates of hydrolysis. As seen with the various $sPLA_2$ tested, when the cells were incubated with lysozyme the activity of the mutant enzymes increased in relation to the wild type. This result confirms that the high charge of the IIA enzyme was required for crossing the outer peptidoglycan layer and not for hydrolysis itself. Because the strategy of charge reversal mutagenesis involved different clusters of cationic residues, see figure 3.3 and (41), it is apparent that there are no specific cationic domains that are required to allow cell wall permeability. The direct involvement of the interfacial surface in cell wall penetration is unlikely based on the results with the 5-site mutant (K53E, R54E, R58E, K124E, R127D) that involves residues distant from the putative interfacial binding surface (84).

The results shown here have for the most part been supported by subsequent findings with *S.aureus* and *Bacillus subtilis* where many of the same charge reversal mutants were used (23). Using *B.subtilis* protoplasts, bacteria where the outer cell wall has been removed, it was confirmed that wild type activity could be restored to the charge reversal mutants and that as seen here many of the mutants showed appreciably higher activity than seen for the wild type enzyme. Rather than using lysostaphin to permeabilise the *S.aureus* as used in this study they attempted to permeabilise their samples by autoclaving. Using this method they too showed an enhancement of phospholipid

hydrolysis however they did not restore the levels of activity to those shown here, suggesting that their method of permeabilisation was not as effective.

The enhanced ability of the wild type hgIIA enzyme to hydrolyse bacterial cell membranes was seen to be paralleled by the enzymes' abilities to produce an antibacterial effect on cell suspensions, as the charge on the $sPLA_2$s reduced so did the ability of these enzymes to kill bacteria. Once again these results have, in the main, been supported by the subsequent work of Koduri *et al.* (23) where they showed using a variety of human and mouse $sPLA_2$ that the IIA enzyme was the most potent tested. Using charge reversal mutants, as shown here, they also confirm that it is the overall charge on the protein that is the dominant factor in its bactericidal abilities.

The importance of the basic nature of the gIIA enzyme to its bactericidal role has also been demonstrated using mutants of *S.aureus* (93). Two mutations that prevent specific modifications of the cell wall (dltA-) and cell membrane (mprF-) polyanions were compared for their effect on cell binding, membrane hydrolysis and antibacterial activity. As discussed previously, section 3.1, bacteria reduce the effectiveness of harmful cationic agents, by reducing their own negativity. These mutants are less able to reduce this negativity and have shown themselves consequently to be more susceptible to hgIIA $sPLA_2$.

It was shown that the reduction in negative charge on the cell wall (lipo)teichoic acids was more important than a reduction in the charge on the membrane itself. This supports evidence presented here where the wild type enzyme was seen to be less active on permeabilised bacterial cells and DOPG SUVs than mutant enzymes possessing a lower charge. The enzyme could be said to pay a "catalytic price" in its anionic membrane hydrolysing capabilities due to its cationic nature, a characteristic necessary for access to these membranes. Indeed studies with *M.luteus* in the presence of varying salt concentrations show that the enzyme's ability to cross the cell wall is dependent on electrostatic interactions easily disrupted by high salt. When the cell wall is permeabilised with lysozyme and treated with increasing salt the enzyme initially becomes more active. As discussed in section 3.2.7, the

molecular basis of this activation is unknown but must reflect multiple electrostatic interactions with the membrane surface leading to changes in either protein or membrane conformation. Koprivnjak *et al.* (93) showed that the charge present on the IIA enzyme was required for the initial binding to the cell wall and that the polyanionic properties of the cell wall facilitated the enzymes transport across the cell wall and access to the underlying membrane.

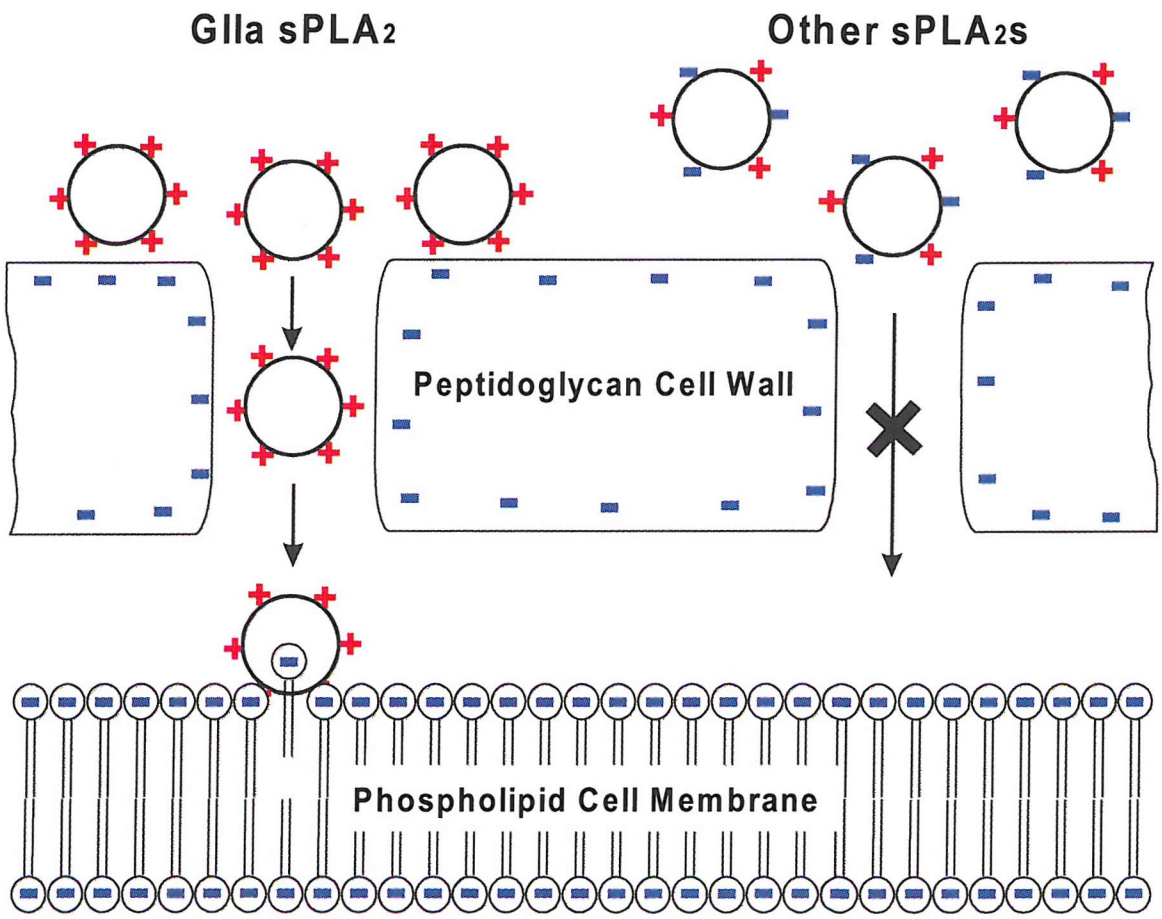


**Figure 3.16** **Model of bacterial cell wall penetration and phospholipid hydrolysis by gIIA $sPLA_2$**

The model proposed for the role of the cationic charges of human group IIA $sPLA_2$ in penetrating the cell wall and gaining access to the underlying cell membrane is shown in Figure 3.16, adapted from (113). Multiple electrostatic interactions between the enzyme and the bacterial cell wall would both increase the local concentration of enzyme at the cell surface and promote its

passage through anionic pores as a result of the continuous making and breaking of electrostatic bonds. Overall, the very high affinity of this enzyme for the anionic phospholipid interfaces would promote the net transfer of enzyme from the cell wall to the bacterial cell membrane.

In the case of Gram-negative bacteria such as *E.coli*, the enzyme has to first penetrate the lipopolysaccharide coat. The pioneering work of Elsbach, Weiss and colleagues has already highlighted the role of BPI in this penetration (54;55) where subsequent work showed that it increased the permeability of the lipopolysaccharide coat (91;92). This enhanced permeability, as seen here with polymyxin B sulphate, allows access of the enzyme to the underlying peptidoglycan cell wall where the selective permeability of $sPLA_2$s can be expressed. In their early studies Elsbach and Weiss showed that the effects of BPI treatment were specific for high pI $sPLA_2$s and the results shown here demonstrate that this selectivity is produced by the anionic peptidoglycan cell wall exposed with BPI and as seen here polymyxin B treatment.

Further studies led them to propose that amino-terminal basic residues in the gIIA enzyme were important in the BPI dependent hydrolysis (54;85). The Gram-positive results discussed above and the initial findings with Gram-negative *E.coli* reported here would question the validity of this statement. The fact that charge reversal of amino-terminal residues on the electrostatically sided pancreatic enzyme increased its BPI dependent hydrolysis might overemphasise the importance of specific amino-terminal residues. Two charge reversal mutants of hgIIA $sPLA_2$ were used here, the amino-terminal and interfacial R7E/K10E and the interfacially remote 5-site mutant K53E/R54E/R58E/K124E/R127D. The results suggest that as seen with the Gram-positive bacteria it is likely to be the global nature of the charge rather than specific amino-terminal or interfacial residues that are important in cell wall permeability. With its high pI, hgIIA would appear to be most suited of the $sPLA_2$ identified to date of expressing significant Gram-negative antibacterial activity *in vivo* in concert with other activating factors found in the extra-cellular environment. It should be noted however that these Gram-negative studies

are at an early stage and much more work will be required before a definitive statement can be made.

Recent transgenic studies in mice expressing the human group IIA enzyme confirm the *in vivo* importance of this enzyme as part of the antibacterial strategy of the organism (63;64). Thus further studies are required to investigate the synergism that this enzyme exhibits with various factors involved in the innate immune response before the role of the hgIIA enzyme can be fully understood.

# Chapter 4 – Interfacial Tryptophan Mutants of Human Group IIA $sPLA_2$

## 4.1 Introduction

An increasing number of 14 kDa human $sPLA_2$ have been discovered (17) but as yet no clear understanding of the physiological roles of these enzymes has emerged (3). The most widely studied member of this human family is the group IIA enzyme where at least part of the function of this mammalian enzyme is that of an acute phase protein with anti-microbial properties, particularly against Gram-positive bacteria (77;56) (see Chapter 3). A direct role in the inflammatory response linked to more long-term arachidonic release is also indicated, a process that involves internalisation of the enzyme (94;95). However, transgenic studies in mice do not appear to support a major role for this enzyme in the inflammatory response linked to arachidonic release and eicosanoid formation (50;96).

One major problem in providing a molecular connection between membrane hydrolysis by this enzyme and arachidonic release is that the human group IIA enzyme is almost inactive against the external surface of the plasma membrane (48;84), the anticipated site of action of this secreted enzyme. This lack of activity is paralleled *in vitro* using PC substrates. In contrast the enzyme expresses high activity against anionic vesicles such as PG and more importantly against bacterial membranes, where labelled *E. coli* membranes provided the best radioactive substrate for the *in vitro* assay of this enzyme.

There are two characteristics that dominate the human group IIA $sPLA_2$'s surface. Firstly, it is a highly cationic protein due to an excess of positively charged residues (see figure 1.5) and this charge is essential for penetrating the anionic cell wall of Gram-positive bacteria. Secondly, it possesses a lack of interfacial tryptophan residues, a property reflected in its inability to hydrolyse phosphatidylcholine vesicles (41), mammalian cell membranes (97) and serum lipoproteins (98). These two properties taken together are entirely consistent with the enzyme being an acute phase antibacterial protein. Such an enzyme would be expected by necessity to be inactive on innate cell membranes and lipoproteins under conditions of acute infection when serum

levels can rise 1000-fold. This inability to hydrolyse zwitterionic membranes is assumed to be due to the protein's inability to bind productively to such condensed neutral interfaces, due in part to a lack of tryptophan residues on its putative interfacial binding surface (12;48).

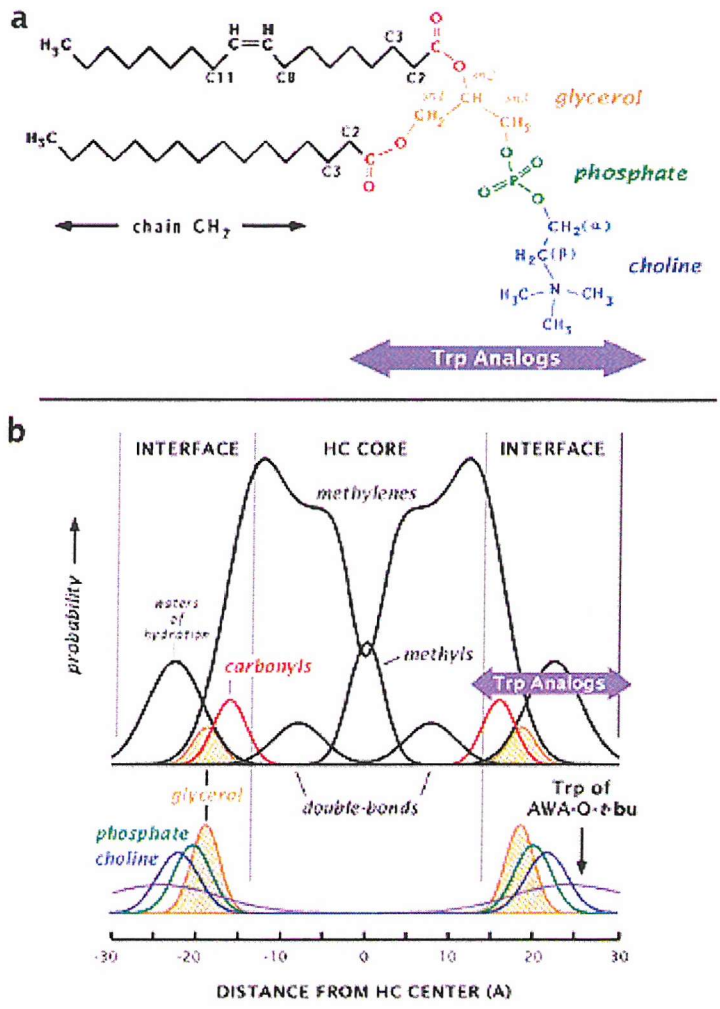


**Figure 4.1** **Structure of POPC (a) and a fluid-phase DOPC bilayer (b)**

**(a)** Chemical structure of POPC molecule and **(b)** Liquid-crystallographic structure of a fluid-phase DOPC bilayer. The resolved image consists of time-averaged transbilayer distributions of the principal lipid groups and water. The lipid region with the strongest interactions with Trp analogues is shown as broad arrows, also shown is the distribution of a Trp residue from Ala-Trp-Ala-O-*tert*-butyl. Taken from (11) with permission.

Tryptophan is found relatively infrequently (1-3 residues) in soluble proteins and appears more frequently in membrane proteins where it tends to reside in the interfacial region of the bilayer (11;99). Where tryptophans are found in

soluble proteins they prove to be important in protein folding, protein-protein, protein-ligand interactions or membrane binding domains (100). When presented as part of a membrane binding domain or site, tryptophan residues have been shown to exhibit a preference for residing in the electrostatically complex interfacial region of phosphatidylcholine bilayers (11) and consequently act as anchors to help position proteins at or within these interfaces (101).

The electrostatically complex interfacial region of a phospholipid bilayer is approximately 1.5nm wide and constitutes the region on each side of the bilayer from the glycerol backbone to the headgroup (102-104). It has been demonstrated using PC bilayers that they are not a stationary planar/slab membrane but that a considerable amount of transbilayer thermal motion (104) is present and that consequently the interfacial region is a complex mixture of components such as methylene, carbonyl, glycerol, phosphate, lipid headgroups and water (figure 4.1).

The aromatic residues tryptophan, tyrosine and phenylalanine possess a permanent quadropole moment due to the presence of π electrons above and below their aromatic rings. The presence of this diffuse electron cloud provides these residues with the ability to participate in a variety of polar interactions including strong cation-π, oxygen-aromatic, sulphur-aromatic, amino-aromatic, and aromatic-aromatic stacking interactions (102). Several such interactions are possible when aromatic residues are situated in the highly heterogeneous lipid-water interfacial region of bilayers.

The larger indole side chain of tryptophan possesses a much more intense region of negative electrostatic potential than tyrosine or phenylalanine and in addition has a strong dipole moment and hydrogen bonding capability created by its imino group (102). It would seem that the large rigid aromatic structure, with both considerable hydrophobic and polar characteristics makes tryptophan partition preferentially into the interfacial region of lipid-water interfaces (11;102;105;106), and therefore ideally suited to contribute to the binding properties of interfacial enzymes.

The importance of tryptophan residues to the interfacial binding of $sPLA_2$s in particular has been demonstrated with the pancreatic group IB enzyme (70;107), the *Naja naja* group IA enzyme (13) and the more recently discovered human group V (20;46) and X enzymes (108;109).

Initial studies with the bovine pancreatic group IB enzyme demonstrated the importance of the W3 tryptophan to the zwitterionic membrane hydrolysis by mutating this residue to an alanine and observing a concomitant loss in activity (110). Subsequently Cho and co-workers studied the role of surface hydrophobic residues on the IB enzyme (70). They were able to demonstrate that by inserting a tryptophan residue in place of a membrane interacting leucine (Leu20) that the activity of the enzyme towards a neutral phospholipid interface could be enhanced by some 2-fold. Insertion of a tryptophan at position Leu19, lining the entrance to the active site slot, interfered with substrate binding demonstrating that the position of the insertion was important and that the residue being replaced needed to interact with the membrane.

*Naja naja* group IA is one of the most active enzymes in terms of its ability to hydrolyse neutral vesicles and cell membranes. The enzyme contains six aromatic residues on its interfacial binding surface and the role of these residues have been assessed by mutating each in turn to an alanine (13). All but one residue (Trp18) made significant contributions to interfacial catalysis. Trp18 like Leu19 of the pancreatic enzyme is positioned at the entrance to the active site and is suggested to be involved in the substrate binding and selectivity of the enzyme. Two tryptophans were of particular importance Trp19 and Trp61 and the removal of these residues had a marked impact on the activity of the enzyme (13).

Since its cloning and recombinant expression in the late 1990s the human group V enzyme has received considerable attention from researchers as it has a more apparent pro-inflammatory role than the closely related group IIA enzyme, including an ability to hydrolyse zwitterionic interfaces such as the

plasma membrane (19). This ability has again been shown by alanine insertion to be due largely to the presence of a tryptophan residue at position 31 on its interfacial binding surface (46).

Very recent work by Gelb and co-workers (108;109) comparing the human group X enzyme to the group IIA and other $sPLA_2$ has demonstrated once more the dominance of tryptophan residues in promoting catalysis of neutral interfaces and their findings will be discussed in more detail later, section 4.3.

The contributions of ionic, aliphatic and aromatic residues to interfacial binding have been compared using Surface Plasmon Resonance (SPR) and a variety of phospholipases (101). It was found that cationic residues accelerated membrane association, aliphatic residues slowed dissociation sometimes penetrating the hydrophobic core of the membrane and that aromatic residues were able to contribute to both association and dissociation steps. The relative contributions of aromatic residues to these interactions was dependent on the chemical nature and orientation of the aromatic side chain as well as its location on the interfacial binding surface (101). As a result of these studies they proposed a general model for interfacial binding of peripheral proteins. They suggested that electrostatic interactions by both ionic and aromatic residues initially brought the protein to the membranes surface and that the subsequent membrane penetration and hydrophobic interactions by aliphatic and aromatic residues stabilised the membrane-protein complexes, thereby elongating the membrane residence time of the protein (101).

Work from this laboratory using an interfacial tryptophan mutant (V3W) of the group IIA enzyme (47;48) has provided further evidence that inserting a tryptophan into the interfacial binding surface can produce an enzyme with enhanced activity on highly condensed zwitterionic interfaces. The lack of tryptophan residues from hgIIA $sPLA_2$ presents an opportunity using site-directed mutagenesis to use inserted tryptophan residues firstly to investigate the effect of this aromatic residue on binding to neutral interfaces and secondly as a reporter group to monitor interfacial binding. Consequently tryptophan residues have been inserted at various positions within the

hydrophobic collar (L2, V3, A19, L20, F24, V31, F70, and Y119 using the pancreatic $PLA_2$ numbering system) surrounding the active site entrance on the putative binding surface of the protein (41) (see figure 4.2).

Although initially made for use as a reporter group, mimicking the Trp3 of the pancreatic group IB enzyme used for such purposes (111), the N1A,V3W mutant of hgIIA showed a remarkable activation on phosphatidylcholine interfaces and mammalian cell membranes compared to the relatively inactive N1A wild type mutant (47;48). The major part of this chapter will be spent expanding on this early work and investigate in more detail the possibility of altering the ability of the enzyme to hydrolyse cell membranes and the basic physiological characteristics of the protein by simple tryptophan insertion.

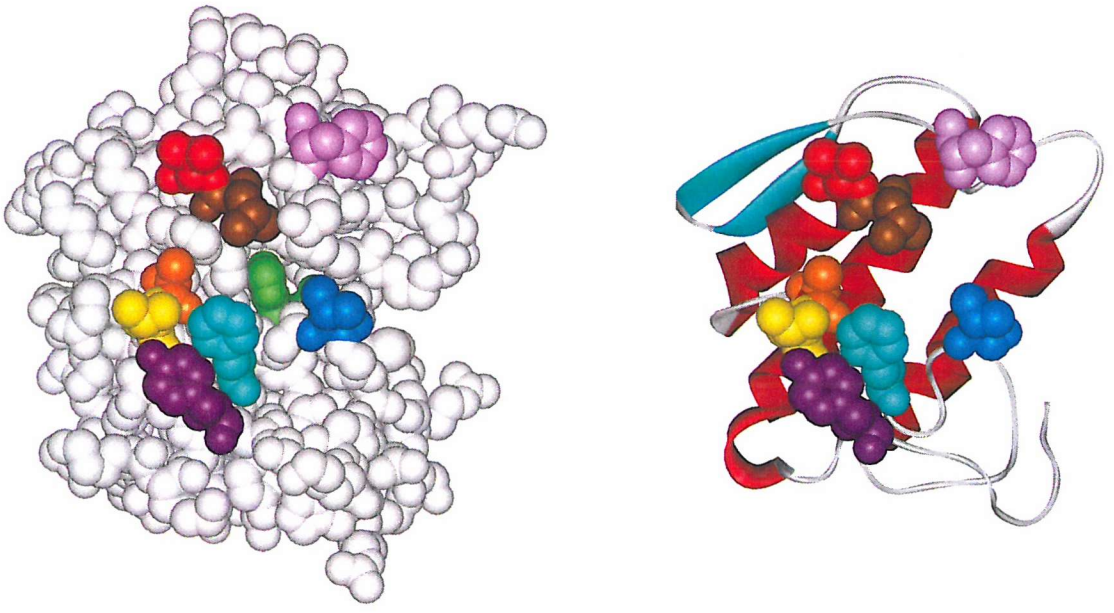

**Figure 4.2** **Hydrophobic Collar of Interfacial Binding Surface**

Hydrophobic residues; brown – L2, red – V3, orange – A19, yellow – L20, cyan – F24, blue – V31, pink – F70, purple – Y119 according to the pancreatic numbering system. The images are shown with the interfacial binding surface pointed towards the viewer; on the left in space filling representation with the catalytic site histidine shown in green and on the right in ribbon form.

Human group IIA $sPLA_2$ is the most extensively studied $PLA_2$ due to its early discovery and suggested role in inflammation, however recent work with the human group V enzyme has highlighted a number of common structural features between these two enzymes. An important similarity between the human group V and IIA enzymes is that both enzymes have a high affinity for

heparin and recently heparin binding has been implicated in the internalisation of these enzymes into some cell types (95;112;113). In particular, the group V $sPLA_2$ has recently been shown to be internalised into HEK293 cells and locate to the perinuclear membrane (20). This phenomenon required both plasma membrane hydrolysis and HSPG binding while, once internalised, targeting to the perinuclear membrane may involve the presence of the interfacial tryptophan. In the case of the C2-like domain of 5-lipoxygenase the targeting of this domain to the PC-rich perinuclear membrane has been attributed to specific tryptophan residues (114) and this subject has been reviewed (115).

The primary focus of this study regarding the potential enhancement of cell membrane hydrolysis and physiological activation by tryptophan insertion has been those residues that are in the same position as critical tryptophan residues found occurring naturally within the hydrophobic collar in other $sPLA_2$s. These critical residues are at positions that have been shown to make a significant contribution to the hydrolysis of zwitterionic interfaces. These positions include W3 from the pancreatic group IB enzyme (110) and W31 from the group V human $sPLA_2$ (46). In particular, the hypothesis that has been explored is that it should be possible to mutate the HSPG-binding group IIA enzyme by tryptophan insertion to produce an enzyme with the basic physiological characteristics of the closely related group V enzyme. In addition it was of interest to see if tryptophan insertion into the group IIA enzyme at the position equivalent to W19 of the venom enzyme (13), namely the L20W mutant, provided the human enzyme with any venom-like properties. Venom enzymes have an enhanced ability to hydrolyse zwitterionic vesicles and cell membranes; they are described as being "highly penetrating" enzymes.

The second aim of this chapter will utilise the intrinsic fluorescence properties of the interfacial tryptophan mutants as a means of reporting on the different environments found at each individual inserted position with an aim to describe the binding properties of this protein in relation to phospholipid bilayers. Tryptophan residues are efficient fluorescent probes as their fluorescence properties are greatly affected by their local environment. The

fact that these fluorescence properties are altered in a largely predictable way by different chemical environments means that these changes can be used as a powerful tool to study the environment of this residue in the free enzyme and when bound to an interface. The wavelength emission maximum of tryptophan residues is considered to be one of the most reliable indicators of their chemical environment and the fluorescence quenching of tryptophan by a variety of molecules has proven to be a useful method of probing residue position and accessibility.

For simplicity all the tryptophan mutants for example N1A,L2W will be referred to as the L2W mutant etc as all these mutants carry the original N1A mutation needed for efficient expression in *E.coli*, and the N1A mutant will be referred to as native or wild type.

## 4.2 Results

### 4.2.1 Characterisation of tryptophan mutants

The interfacial tryptophan mutants L2W, A19W, L20W, F24W, V31W, F70W, Y119W, and V3,31W of human group IIA $sPLA_2$ were prepared using PCR site-directed mutagenesis. They were expressed and purified using the same method and conditions as for the original N1A (wild type) and V3W enzymes (see section 2.3.2) (47;48). The yields for these mutants were all similar to those of the N1A (wild type) mutant with the exception of L2W, which expressed at a much lower level. This low-level expression was paralleled by a L2C mutant of hgIIA prepared at the same time by another member of the lab (N. Giles personal communication). Analysis by SDS-PAGE (figure 4.3) has shown all the mutants to be pure once subjected to cation exchange and heparin affinity column chromatography on an FPLC (see 2.3.2.4 and 2.3.2.5) and that they migrated at approximately 17kDa with the N1A (wild type) protein.

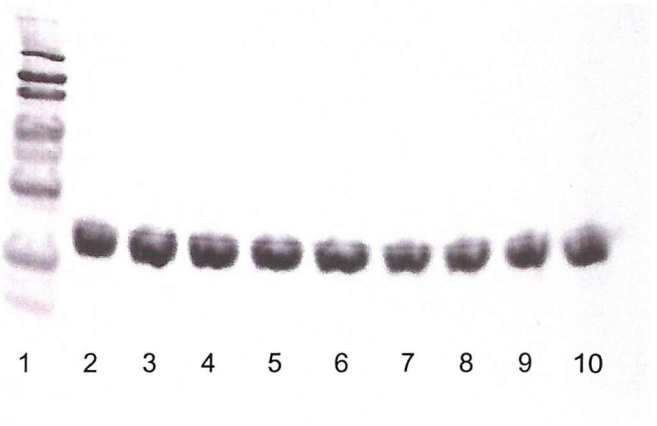


**Figure 4.3** **SDS-PAGE of purified interfacial tryptophan mutants of hgIIA $sPLA_2$**

Samples were run under denaturing conditions on 15% SDS-PAGE. Lane 1 – molecular weight markers, 2 – wild type, 3 – L2W, 4 – A19W, 5 – L20W, 6 – F24W, 7 – V31W, 8 – F70W, 9 – Y119W, 10 – V3,31W, as described in Materials and Methods. Molecular weight markers (from top) 66, 45, 36, 29, 24, 20, 14.2 and 6.5kDa.

### 4.2.2 Mass Spectrometry

To ensure incorporation of the expected mutation into the recombinant protein the purified samples were subjected to electrospray ionisation mass spectrometry analysis. The results obtained are shown in table 4.1 and figures 4.4-4.6 and confirm the primary structure of the mutants. The original N1A (wild type) mutant was used as a control.

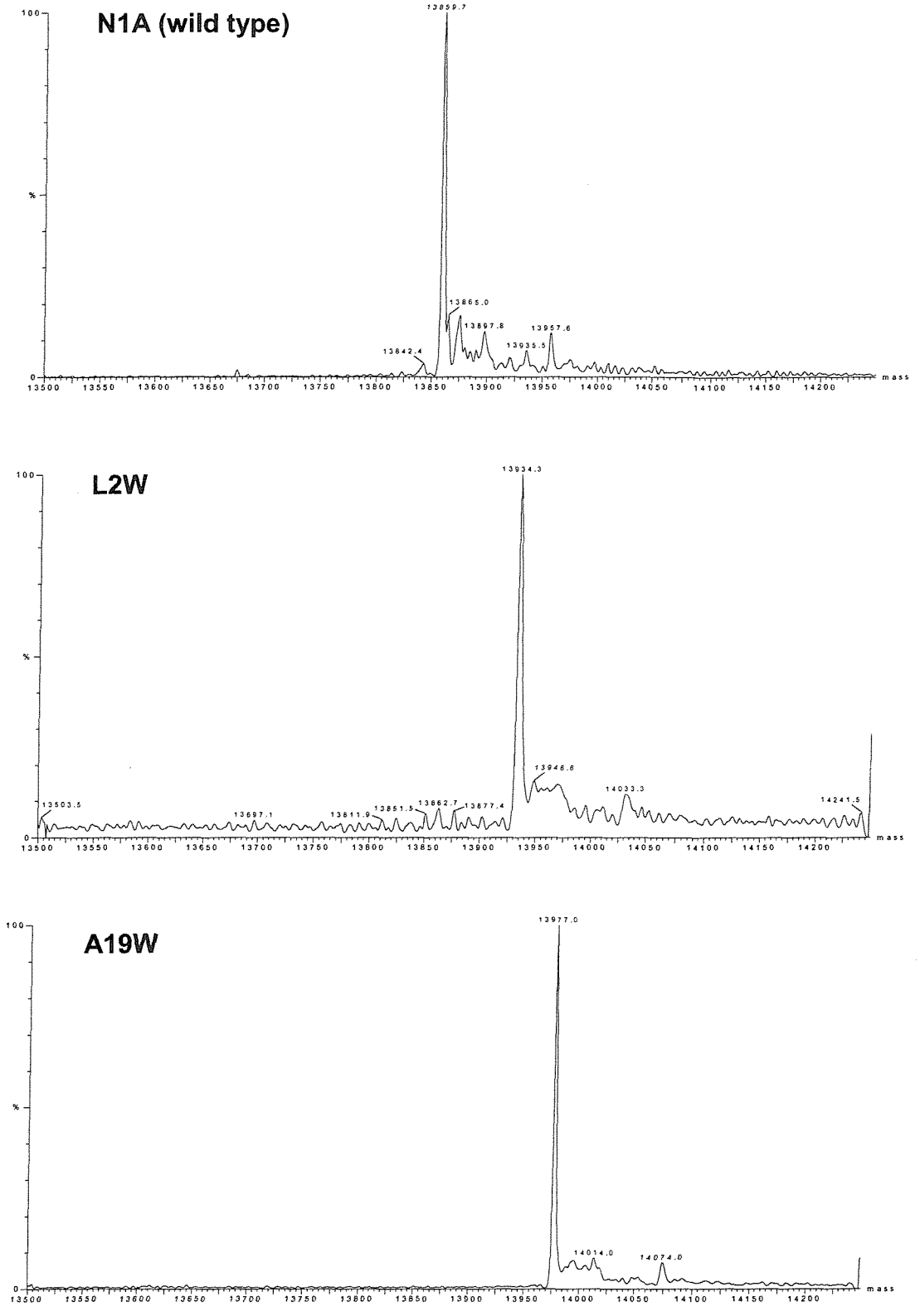


**Figure 4.4** **Mass spectra of N1A (wild type), L2W and A19W mutants of hgIIA $sPLA_2$**

Electrospray ionisation mass spectrometry was carried out as described in Materials and Methods, section 2.4.1.

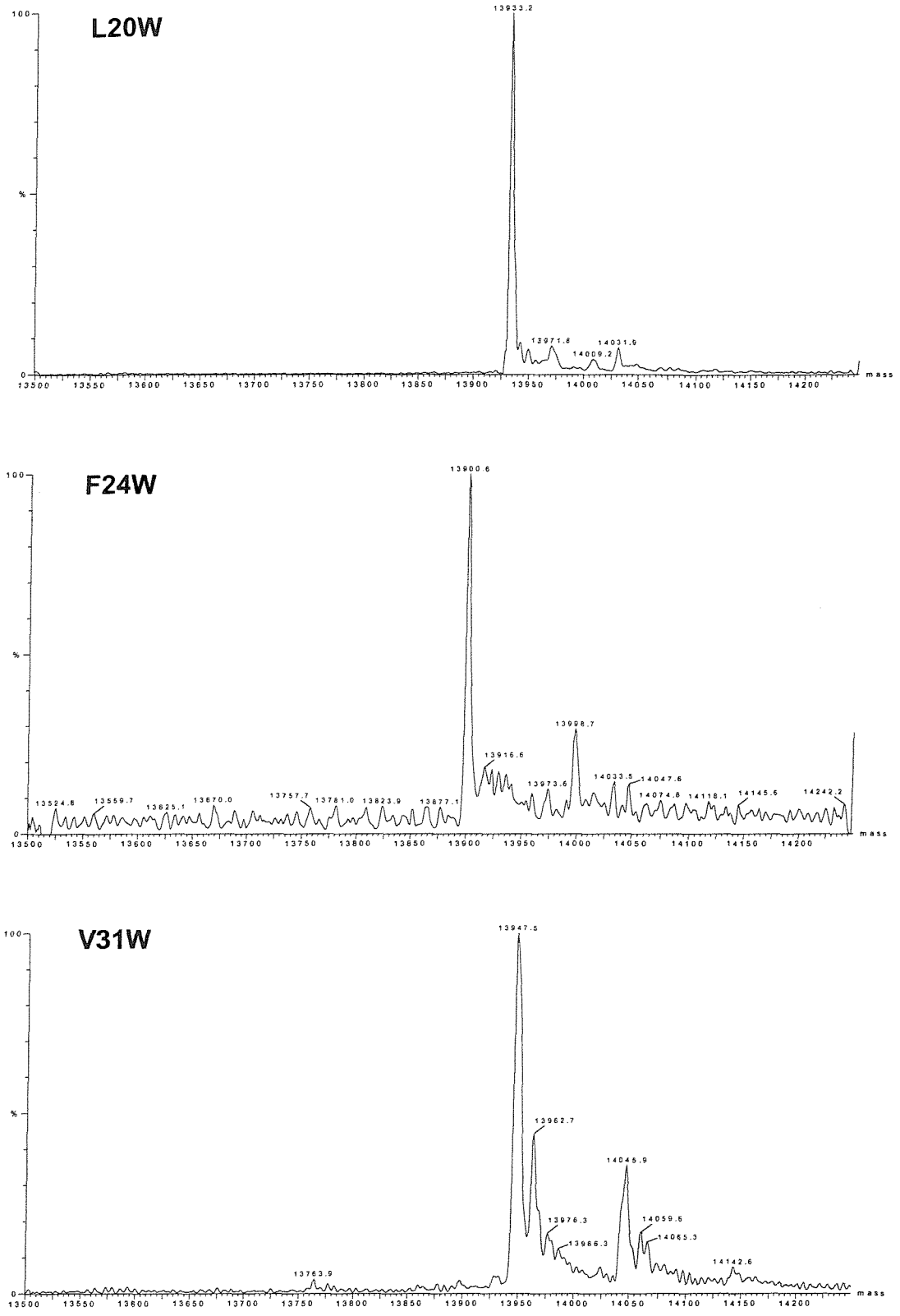


**Figure 4.5** **Mass spectra of L20W, F24W and V31W mutants of hgIIA $sPLA_2$**

Electrospray ionisation mass spectrometry was carried out as described in Materials and Methods, section 2.4.1.

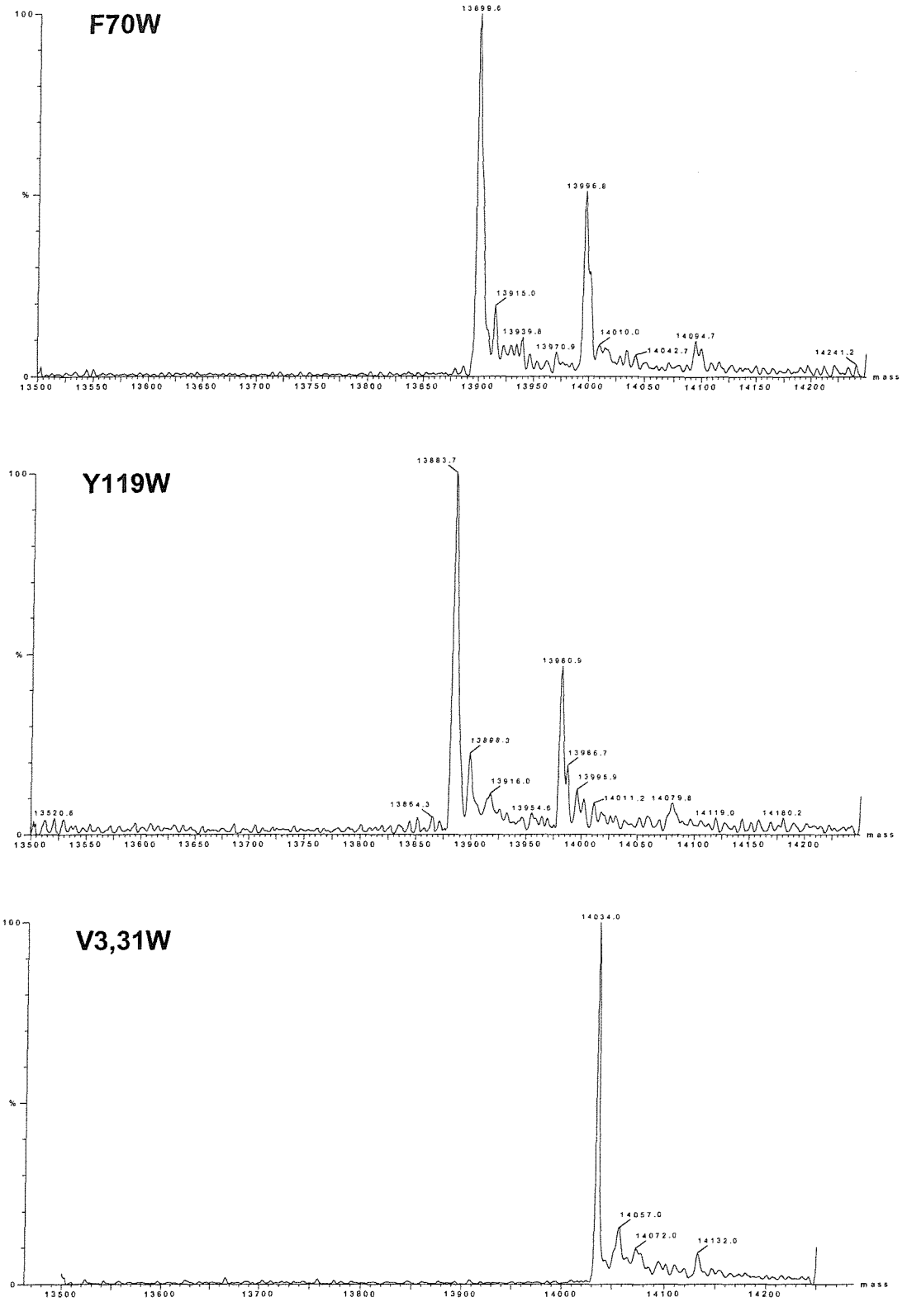


**Figure 4.6** **Mass spectra of F70W, Y119W and V3,31W mutants of hgIIA $sPLA_2$**

Electrospray ionisation mass spectrometry was carried out as described in Materials and Methods, section 2.4.1.

| Mass Spectrometry | | |
|---|---|---|
| | Predicted mass (Da) | Result (Da) |
| Wild type (N1A) | 13860.9 | 13859.7 |
| L2W | 13933.9 | 13934.3 |
| A19W | 13976.0 | 13977.0 |
| L20W | 13933.9 | 13933.2 |
| F24W | 13899.9 | 13900.6 |
| V31W | 13947.9 | 13947.5 |
| F70W | 13899.9 | 13899.6 |
| Y119W | 13883.9 | 13883.7 |
| V3,31W | 14035.0 | 14034.0 |

**Table 4.1** **Mass spectrometry results**

Electrospray ionisation mass spectrometry was carried out as described in Materials and Methods, section 2.4.1.

### 4.2.3 Circular Dichroism

Before further characterisation of the tryptophan mutants could be performed it was important to confirm that the mutations had not altered the structural integrity of the protein and that the proteins were correctly folded. Far UV circular dichroism was used to compare the secondary structure of the mutants to wild type, and the spectra are shown in figure 4.7. No significant differences in structure were indicated for all but one of the mutants, the low-expression L2W mutant. The spectra obtained for this mutant may well reflect the low concentration of the sample used rather than any real perturbation of the secondary structure, however due to the very low quantities of L2W obtained and the limited amount of it available, no clear spectra were obtained on this mutant. Despite this fact the L2W mutant was included in the further analysis of these mutants with a view to gaining a CD spectra of it at a later date should this mutation prove to alter the characteristics of the protein.

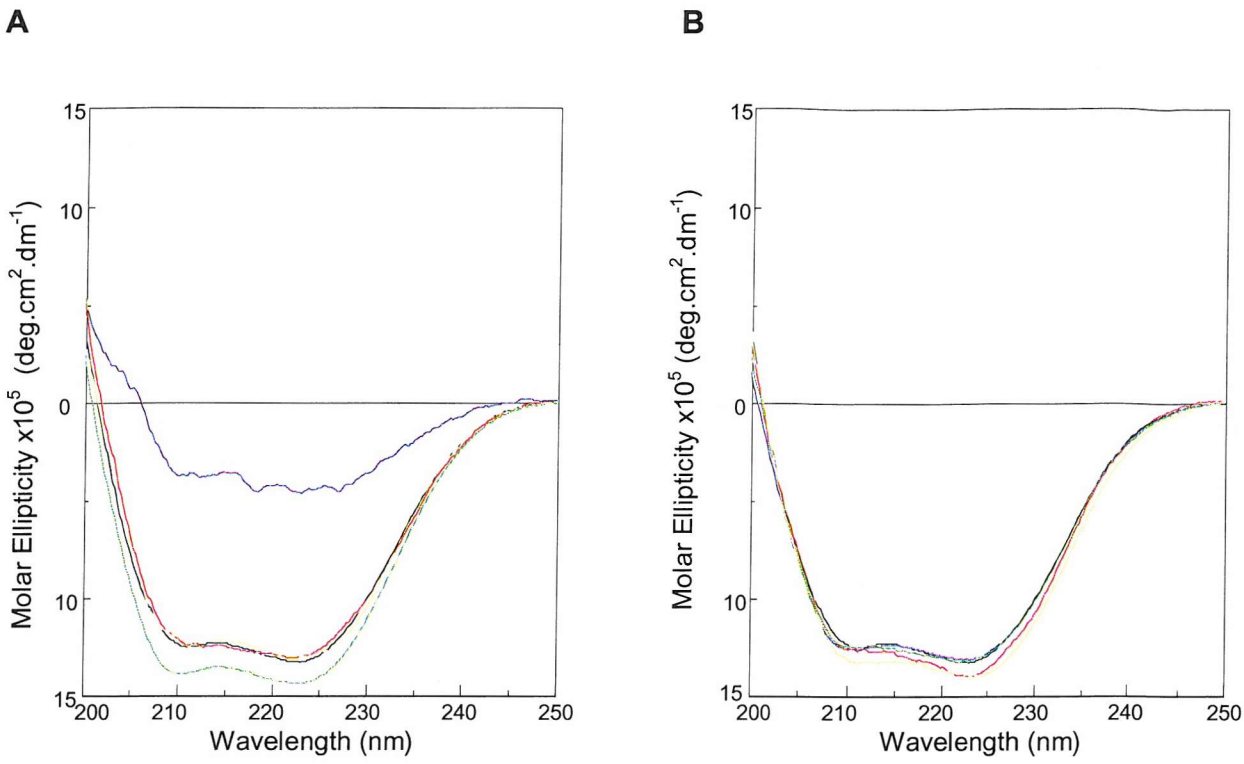


**Figure 4.7** **Circular Dichroism Spectra of N1A and tryptophan mutants**

CD spectra were carried out in 10mM $KH_2PO_4$ **A**; Wild type (N1A) – black, L2W – blue, A19W – red, L20W – green and F24W – yellow. **B**; Wild type (N1A) – black, V31W – blue, F70W – red, Y119W – green, and V3,31W – yellow. Spectra shown are the product of 3 accumulations and were carried out as described in the Materials and Methods.

### 4.2.4 Phospholipid Vesicle Activity Assays

#### 4.2.4.1 Anionic Phospholipid Hydrolysis

All $sPLA_2$s thus far identified bind with high affinity to anionic phospholipid vesicles and can exhibit catalysis in the scooting mode. Under these conditions the enzymes remain attached to the membrane surface for many catalytic turnovers and in the case of the pancreatic group IB $sPLA_2$ can lead to the complete hydrolysis of the outer leaflet of the vesicle (15). Scooting mode kinetics allows for the effective separation of interfacial binding and activation from factors affecting the catalytic event itself. The activities of the various tryptophan mutants were compared to the wild type (N1A) enzyme

under these scooting conditions. The specific activity was determined using DOPG SUVs (formed by methanol injection) and mixed with FABP and DAUDA, so that fatty acid release resulting from phospholipid hydrolysis could be monitored in real time. The tryptophan mutants showed no major difference in specific activity, with the exception of L2W that demonstrated a 5-fold reduction, see table 4.2. A similar reduction with a L2W mutation of the porcine pancreatic group IB enzyme has previously been observed (107). It was proposed that the indole ring of the introduced tryptophan residue, which lines the active site slot, interfered with the orientation of the substrate leading to the observed reduction in specific activity.

| | DOPG SUVs | | DOPG SUVs | | DOPC SUVs | |
|---|---|---|---|---|---|---|
| | 0.1M NaCl % (wild type) | S.D. | 2M NaCl % (wild type) | S.D. | % (V3W) | S.D. |
| Wild type | 100[α] | 31 | 100[β] | 4.4 | 3 | 0.2 |
| L2W | 21 | 1.3 | - | - | 2.6 | 0.1 |
| V3W | 116 | 2.5 | 584 | 8.4 | 100[γ] | 47.9 |
| A19W | 85 | 9.6 | - | - | 2.9 | 0.1 |
| L20W | 95 | 18.1 | 116 | 7.6 | 2.5 | 0.4 |
| F24W | 103 | 5.2 | - | - | 4.1 | 0.1 |
| V31W | 151 | 12.8 | 577 | 18 | 20 | 1.2 |
| F70W | 103 | 5.6 | - | - | 5 | 0.7 |
| Y119W | 119 | 22 | - | - | 5.3 | 0.2 |
| V3,31W | 126 | 16.5 | 600 | 14 | 206 | 6.4 |
| hgV | 25 | 4.7 | - | - | 259 | 14 |
| *Naja naja* | 108 | 9.6 | - | - | 3559 | 636 |

**Table 4.2** **Relative activities of $sPLA_2$ on phospholipid vesicles**

The absolute specific activity values for wild type[α], wild type[β] and V3W[γ] were 96 ± 30, 43 ± 2 and 17 ± 8.2 nmol/min/μg respectively. Specific activities were determined using a fluorescence displacement assay described in (75). Assays were performed in the presence of 62.5μM phospholipid and data are means ± S.D. (n=3).

Due to the structural similarities of the group IB and IIA enzymes the L2W mutation of the hgIIA may well lead to similar effects. However, as suggested by the CD spectra (see figure 4.7) the structural integrity of the L2W mutant

may have been affected by the mutation leading to the observed reduction in activity or there may simply be a concentration problem with this protein sample. It should be noted that the V31W mutant expressed significantly higher activity under these conditions than the wild type enzyme and there is no molecular explanation for this at this time. The fact that all the mutants (with the exception of L2W) displayed similar activity on DOPG SUVs compared with the wild type enzyme confirms that the mutants are structurally intact.

#### 4.2.4.2 Zwitterionic Phospholipid Hydrolysis

The hgIIA $sPLA_2$ is characterised by extremely low activity on zwitterionic interfaces, a property reflecting the enzyme's inability to bind productively to such condensed neutral interfaces. As has been demonstrated previously with the V3W mutant (47;48) the introduction of a tryptophan residue on the interfacial binding surface should have a significant effect on the mutant's ability to bind to and consequently hydrolyse such zwitterionic interfaces. Table 4.2 shows that along with the V3W mutant, which exhibits a 30 to 40-fold increase in activity, only the V31W mutant, with a 7-fold enhancement, shows a significant increase in activity compared with the wild type on DOPC SUVs. The enhancement of activity seen with the V3W mutant on PC (30 to 40-fold) is less than has previously been reported (47;48) and reflects the difficulty in obtaining an accurate value for the specific activity of the N1A wild type enzyme. The wild type enzyme, as discussed previously, is essentially inactive on such interfaces and in order to obtain measurable rates a large amount of the enzyme is required. It would appear that these assay conditions underestimate the specific activity of the enzyme.

Three of the mutated residues are representative of naturally occurring tryptophans in other structurally similar $sPLA_2$s that are active on zwitterionic interfaces and cell membranes. The V3W mutation is representative of the tryptophan found in the pancreatic group IB enzyme, L20W of *Naja naja* group

IA and V31W the human group V enzyme, of these both the V3W and V31W show enhancement on PC interfaces whereas the L20W does not (table 4.2). None of the remaining mutants demonstrated a major enhancement in PC activity while they all show similar activities on PG vesicles. The differences in PC activity observed for the V3W and V31W mutants should reflect differences in the mutants' ability to bind to such interfaces when compared to the other tryptophan mutants and the wild type enzyme.

Because both the V3W and V31W mutants display enhanced activity on PC a double mutant V3,31W was produced. Table 4.2 shows that the tryptophan induced rate increase of the V3,31W double mutant is further enhanced when compared to the wild type enzyme on DOPC vesicles. The activity of the V3,31W mutant now approaches that of the PC-active hgV $sPLA_2$ although it is still very much less active than the double interfacial tryptophan-containing *Naja naja* gIA enzyme. The similar activity of the double mutant V3,31W with the hgV enzyme suggests that this enzyme may now function in a similar manner to the group V enzyme under physiological conditions. The L2W mutant which showed decreased activity on DOPG vesicles shows no enhanced activity with DOPC and due to the difficulty in obtaining even a very limited quantity of this enzyme and the possibility that it is not folded correctly it was not included in the further studies of the tryptophan mutants.

### 4.2.4.3 Effect of Salt on Anionic Phospholipid Hydrolysis

The fact that no large effects are seen on enzyme activity using anionic vesicles as substrate as a result of tryptophan insertion (see section 4.2.4.1 and table 4.2) is consistent with the already high binding affinity of the hgIIA enzyme ($K_d$ ~ $10^{-10}$M) to such anionic interfaces. Under these assay conditions a major contribution to this high affinity comes from electrostatic interactions (41;83;101). The importance of electrostatics to the interfacial binding of hgIIA to anionic interfaces can be demonstrated under conditions of high salt (table 4.2). Continuous displacement fluorescence assays using the

PC enhancing mutants V3W, V31W and the double V3,31W were compared to the wild type and L20W mutant, using DOPG vesicles in the presence of 2M NaCl. The importance of electrostatic interactions is apparent due to the fact that it required such a high salt concentration to minimise the effect of electrostatic interactions and to discriminate between the mutants in terms of potential hydrophobic interactions. Under these conditions an enhanced ability of the V3W, V31W and V3,31W mutants to hydrolyse DOPG SUVs was seen compared to the wild type and L20W mutant. The results highlight the increased hydrophobic contribution to interfacial binding of these mutants resulting in enhanced activity.

### 4.2.5 Binding Studies using Tryptophan Fluorescence Properties

It has been demonstrated that the V3W, V31W and V3,31W mutants have appreciably different characteristics on PC membranes compared with the other tryptophan mutants and wild type enzyme (table 4.2). The fact that the activities for all the hgIIA enzymes were similar on anionic DOPG vesicles demonstrated that mutagenesis has not adversely affected the catalytic potential of these tryptophan mutants. Therefore, the differences in DOPC hydrolysis demonstrated would be expected to reflect a change in binding to the interface.

Using the intrinsic fluorescence of tryptophan residues the environment around the introduced tryptophans has been investigated in free solution and in the presence of DOPG and DOPC SUVs, see figures 4.8 and 4.9 and tables 4.3 and 4.4. The tryptophan mutants were excited at 290nm, to reduce the contributing effects of fluorescence and resonance energy transfer from tyrosine and phenylalanine residues, and their fluorescence emission spectra recorded between 300 and 500nm.

When the emission spectra of the tryptophan mutants in free solution are compared it can be seen that the mutants are exposed to subtly different environments. V3W and A19W are blue-shifted compared to the other mutants

at 346.3 and 346.7nm respectively suggesting a less polar environment reflecting a more buried position. F24W and F70W are intermediately positioned at 350.8nm whereas, L20W, V31W and Y119W are red-shifted at 352.2, 352.3 and 351.8 respectively suggesting that they are fully exposed to the aqueous solution, L-tryptophan in free solution under these conditions had a comparable wavelength maximum of 352.2nm (data not shown). At these red-shifted wavelengths the indole ring of the tryptophan side chain demonstrates a polarity dependent red-shift (116) which will be largely dependent on the residues around each inserted tryptophan and such sequence effects rather than differences in water exposure may explain some of the differences observed. Generally the emission spectra of the tryptophan mutants shown here are red shifted and featureless suggesting that all are exposed to the aqueous solution and therefore probably capable of interacting with phospholipid interfaces.

| Protein | Buffer $\lambda_{max}$ (nm) | DOPG 180μM $\lambda_{max}$ (nm) | $\lambda_{max}$ shift (nm) | Increase in Quantum Yield (%) | DOPC 180μM $\lambda_{max}$ (nm) | $\lambda_{max}$ shift (nm) | Increase in Quantum Yield (%) |
|---|---|---|---|---|---|---|---|
| V3W | 346.3 ± 1.2 | 339.3 ± 0.6 | 7 ± 1 | 28.9 ± 6 | 345.2 ± 1.9 | 1.2 ± 2.6 | -0.1 ± 2.5 |
| A19W | 346.7 ± 0.6 | 330.5 ± 0.5 | 16.2 ± 1 | 5.3 ± 3.6 | 346.5 ± 0.5 | 0.2 ± 0.3 | -3.3 ± 5.2 |
| L20W | 352.3 ± 0.8 | 332 ± 2.3 | 20.3 ± 2.3 | 62.7 ± 6.6 | 352.5 ± 0.5 | -0.2 ± 1.3 | -0.7 ± 2.2 |
| F24W | 350.8 ± 0.3 | 339 ± 0.5 | 11.8 ± 0.8 | 147.1 ± 4 | 349.2 ± 0.6 | 1.67 ± 0.3 | 7.7 ± 4.3 |
| V31W | 353.8 ± 0.8 | 342.7 ± 0.6 | 11.2 ± 1.3 | 144.6 ± 18.8 | 353.5 ± 0.5 | 0.3 ± 0.3 | 1.8 ± 3.5 |
| F70W | 350.8 ± 0.3 | 338.3 ± 0.3 | 12.5 ± 0.5 | 134.9 ± 6.9 | 349.3 ± 0.6 | 1.5 ± 0.9 | 1.3 ± 2.1 |
| Y119W | 352.3 ± 0.3 | 335.3 ± 0.3 | 17 ± 0.5 | 107.5 ± 16.6 | 352 ± 0 | 0.3 ±0.3 | 5.9 ± 10.8 |
| V3,31W | 349.2 ± 0.3 | 341.5 ± 0 | 7.7 ± 0.3 | 86.6 ± 13.7 | 341.5±0 | 7.7 ± 0.3 | 88.4 ± 10 |

**Table 4.3** **Spectral characteristics of tryptophan-containing mutants of hgIIA $sPLA_2$ in the presence of 180μM DOPG or DOPC**

Tryptophan fluorescence spectra were recorded for the enzymes (0.2μM) in buffer 20mM Tris 1mM EGTA, or in the presence of DOPG or DOPC SUVs. The phospholipid concentrations were 180μM and the spectra recorded as detailed in Materials and Methods. The data shown are means ± S.D. (n=3).

The wild type hgIIA $sPLA_2$ has been shown to have a $K_d$ of ~$10^{-10}$M on anionic interfaces (35), therefore in the presence of 180μM DOPG 0.2 μM enzyme could be expected to be fully bound. Under these conditions, as expected from their exposed position on the interfacial binding surface, all of

the tryptophan mutants demonstrated a fluorescence enhancement and blue-shift in their emission wavelength maximum indicating interfacial binding (figures 4.8, 4.9 and table 4.3).

The V3W mutant (figure 4.8A) shows a blue-shift in its wavelength emission maxima on binding of some 7nm to 339.3nm and an increase in quantum yield of 29% showing that it has become desolvated. A19W and L20W (figures 4.9A and 4.8C) show dramatic blue-shifts of 16 and 20nm to 330.5 and 332nm and increases in quantum yield of 5 and 63% respectively. Such emission maxima are similar to that reported for tryptophans present on the non-polar face of a membrane penetrating amphipathic helix oriented parallel to the membrane's surface (331nm) (117) and would suggest such an orientation for this short helix. The V3W mutant due to its position on the interfacial face of the amino-terminal helix with its previously demonstrated importance in interfacial binding, might have been expected to have a more blue-shifted emission maximum in line with the A19W and L20W mutants. In contrast with those mutants which are positioned on helix 2 which is relatively amphipathic in nature, V3W sits on a highly cationic helix and next to His6 with which it could hydrogen bond possibly leading to the observed red-shifted fluorescence.

The F24W, V31W and F70W mutants (figures 4.9C, 4.8E and 4.9E) all display similar spectral changes to each other with blue-shifts of 11.8, 11.2 and 12.4nm to 339, 342.7 and 338.3nm respectively and large increases in quantum yield of 147, 145 and 135% respectively. These relatively red-shifted values when bound would suggest that these residues are situated in a more hydrated region of the bilayer and therefore demonstrate a shallower penetration of the membrane by the protein at these positions. Y119W exhibits a somewhat intermediate characteristic with a dramatic shift in wavelength of some 17nm to 335.3nm and a large increase in fluorescence of some 108%. Finally, as expected the double tryptophan mutant V3,31W exhibits an emission spectra positioned somewhere between the spectra recorded for the single mutants V3W and V31W, that is a blue-shift of 8nm to 341.5nm and an increase in fluorescence yield of 87%.



These results demonstrate that all the residues are affected by interfacial binding, as expected, but that the fluorescence shifts observed may reflect the insertion of the protein into the bilayer at different positions around the active site slot.

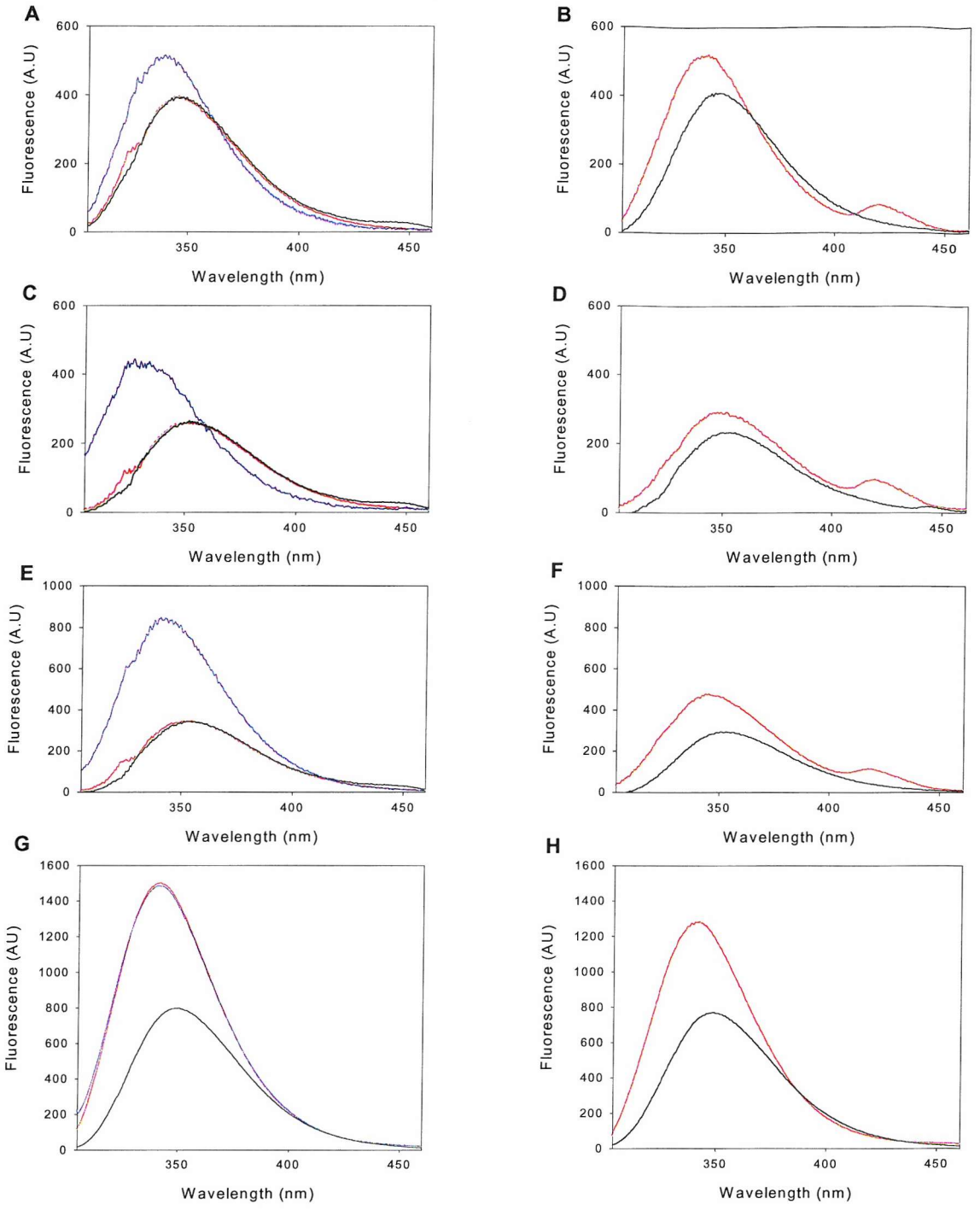


**Figure 4.8** **Fluorescence emission spectra of V3W (A&B), L20W (C&D), V31W (E&F) and double mutant V3,31W (G&H)**

The enzymes (0.2μM) are shown in buffer 20mM Tris 1mM EGTA (black line), DOPG (blue lines) and DOPC (red lines). The phospholipid concentrations were 180μM (A, C, E and G) and 1mM (B, D, F, and H). Excitation was at 290nm. Data shown are means ± S.D. (n=3).

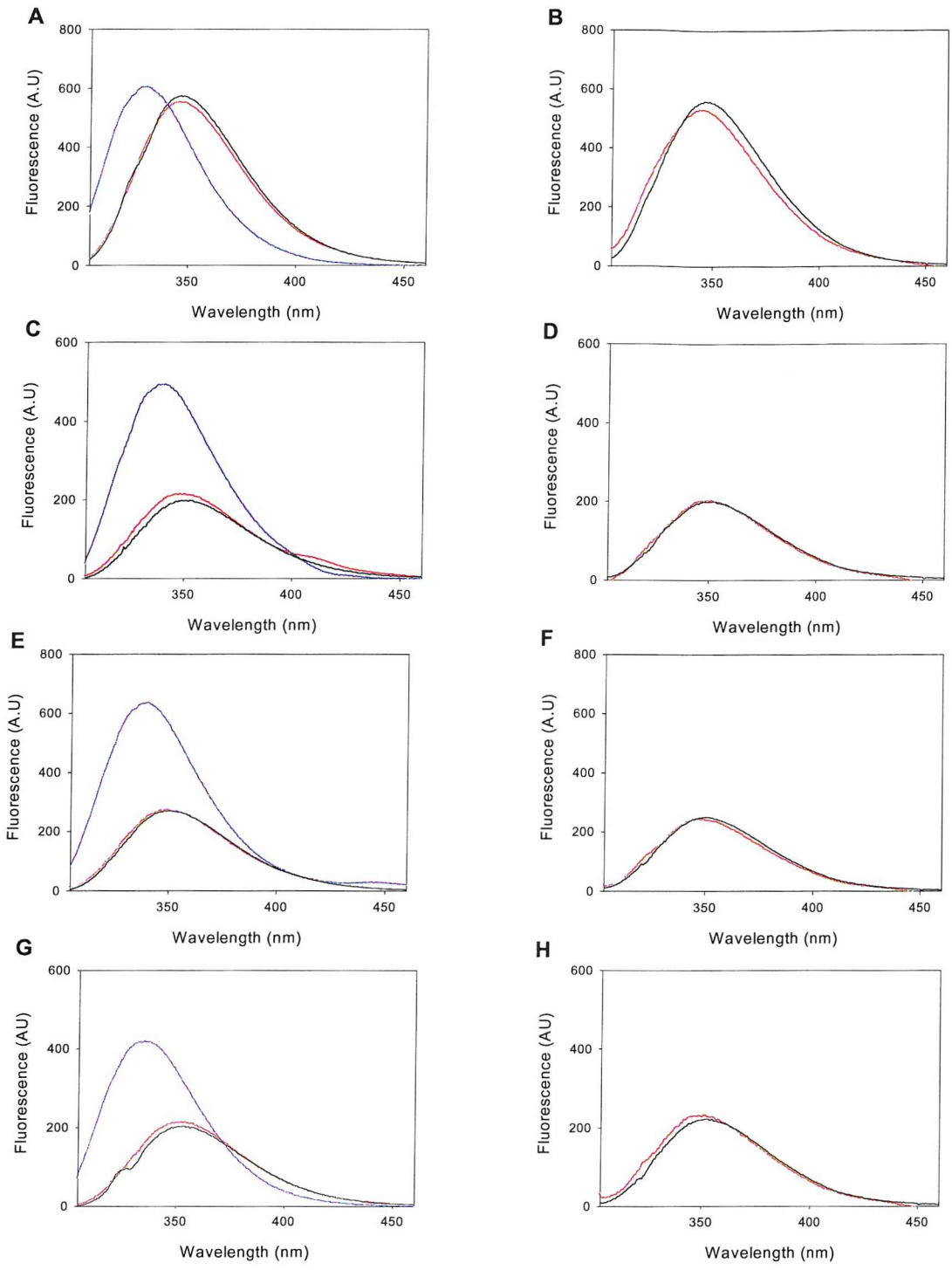


**Figure 4.9** **Fluorescence emission spectra of A19W (A&B), F24W (C&D), F70W (E&F) and Y119W (G&H)**

The enzymes (0.2μM) are shown in buffer 20mM Tris 1mM EGTA (black line), DOPG (blue lines) and DOPC (red lines). The phospholipid concentrations were 180μM (A, C, E and G) and 1mM (B, D, F, and H). Excitation was at 290nm. Data shown are means ± S.D. (n=3).

When the fluorescence emission spectra of the mutants were recorded in the presence of 180μM DOPC SUVs, none of the single mutants exhibited a significant shift in emission maximum suggesting that no detectable binding is seen at this concentration of DOPC. In the earlier activity assays (table 4.2) the amount of activity observed with the V3W mutant on DOPC might be expected to reflect less than 10% of the enzyme binding. It should therefore be no surprise that at 180μM PC little or no binding is visible with the PC active V3W mutant or the less active V31W. In marked contrast the V3,31W double mutant which demonstrated relatively high activity on DOPC can be seen to demonstrate a virtually identical fluorescence change at 180μM DOPC when compared to the same concentration of DOPG see figure 4.8G and table 4.3 suggesting that even at this level of DOPC the double mutant is fully bound.

| Protein | Buffer $\lambda_{max}$ (nm) | DOPC 1mM $\lambda_{max}$ (nm) | $\lambda_{max}$ shift (nm) | Increase in Quantum Yield (%) |
|---|---|---|---|---|
| V3W | 346.7 ± 0.3 | 340.5 ± 0.5 | 6.2 ± 0.3 | 27.2 ± 10.5 |
| A19W | 346.8 ± 0.3 | 345± 0.5 | 1.8 ± 0.3 | -4.5 ± 6.5 |
| L20W | 352.2 ± 0.3 | 350.3 ± 0.3 | 1.8 ± 0.3 | 24.6 ± 10.8 |
| F24W | 350 ± 0.5 | 349.8 ± 0.3 | 0.2 ± 0.3 | 1.4 ± 3.3 |
| V31W | 352.3 ± 0.8 | 346 ± 0.9 | 6.3 ± 1.6 | 63.6 ± 10.7 |
| F70W | 350 ± 0 | 349 ± 0.5 | 1 ± 0.5 | -2.5 ± 7.8 |
| Y119W | 351.8 ±0.3 | 350 ± 0 | 1.8 ± 0.3 | 4 ± 12.5 |
| V3,31W | 348.3 ± 0.6 | 341.2 ± 0.3 | 7.2 ± 0.3 | 66.3 ± 7.6 |

**Table 4.4** **Spectral characteristics of tryptophan-containing mutants of hgIIA sPLA$_2$ in the presence of 1mM DOPC**

Tryptophan fluorescence spectra were recorded for the enzymes (0.2μM) in buffer 20mM Tris 1mM EGTA, or in the presence of 1mM DOPC SUVs. The spectra were recorded as detailed in Materials and Methods and the data shown are means ± S.D. (n=3).

In an attempt to distinguish between the single tryptophan mutants with regard to their PC binding abilities the DOPC concentration was increased to 1mM and the spectra recorded as before. The results are shown in figures 4.8 and 4.9B, D, F, and H and table 4.4. Under these conditions V3W (figure 4.8B) has a blue-shift in its wavelength emission maximum of 6nm and an increase in quantum yield of 27% representing levels similar to that seen with DOPG.

V31W (figure 4.8F) shows a shift of 6.5nm and an increase in quantum yield of 62% again approaching levels seen with DOPG. The other single tryptophan mutants exhibit modest changes in their fluorescence spectra under these conditions with small blue-shifts of 2nm for A19W, L20W and Y119W and no real changes with F24W and F70W. These results suggest that in accordance with the activity data V3W binds more tightly than V31W to DOPC vesicles with the remaining single mutants exhibiting minimal binding at PC concentrations of up to 1mM. In the presence of 1mM DOPC the V3,31W double mutant demonstrates an identical blue-shift in its emission maximum but with a slightly lower increase in quantum yield.

These results taken together suggest that the PG results obtained represent the fluorescence spectra of each tryptophan when the protein is fully bound to an interface. The extent to which the PC spectra approach these 'fully bound' spectra is a reflection of the extent of binding of that mutant at that particular concentration. Although the above results demonstrate that the V3,31W double mutant is fully bound to PC it demonstrates a significantly lower specific activity on DOPC compared to DOPG SUVs (table 4.2) and this could be due to a number of physical factors related to vesicle structure. However it is noteworthy that the hgIIA enzyme has lower substrate specificity for PC as opposed to PG headgroups. In assays involving vesicles containing equi-molar ratios of PC and PG and using ESI-MS to analyse product formation, the enzyme showed a 3 to 4-fold preference for PG over PC (E.L.Heeley, A.D.Postle and D.C.Wilton, unpublished data) but this may simply reflect $K_m$ rather than $K_{cat}$ differences.

### 4.2.6 Fluorescence Quenching of tryptophan mutants by acrylamide

The sensitivity of tryptophan fluorescence to its local environment means that tryptophan residues within a protein have a further property that may be exploited; that is fluorescence quenching. Fluorescence quenching refers to any process that leads to the reduction in intensity of emission from a fluorophore. Tryptophan fluorescence can be decreased by a wide variety of

small molecules and ions through static and collisional quenching. Both static and collisional quenching rely on molecular contact between the fluorophore and the quencher, thus the amount of quenching seen in a given instance will be related to the accessibility of the fluorophore to that quencher. The accessibility of a tryptophan residue to such quenchers can be used to determine the location of a tryptophan within a protein or in relation to an interface.

Acrylamide is an electron-deficient molecule that is believed to quench tryptophan fluorescence in the excited state by electron transfer from the indole ring of tryptophan (118). Acrylamide was chosen for this investigation as it is a neutral quencher that has limited accessibility to phospholipid bilayers. This property was used to probe the tryptophan mutants free in solution and in the presence of DOPG and DOPC SUVs. Acrylamide as a neutral molecule should be less affected by the high positive charge on the proteins surface or by the difference in charge present on the two interfaces to be investigated (PG is anionic and PC is neutral) than charged quenchers such as iodide. The tryptophan mutants produced here, situated on the interfacial binding surface of the protein, would be expected to be accessible to quenching by acrylamide when free in solution and become protected on binding to an interface.

Figure 4.10A shows quenching carried out with the 3 mutants representative of naturally occurring tryptophans in other $sPLA_2$. Clearly, at a concentration of 0.2μM all three of the tryptophan mutants are considerably less accessible to acrylamide in the presence of 180μM DOPG than when free in solution. This reduced accessibility suggests that the tryptophan residues are protected by the binding process, consistent with the blue-shifts seen with the fluorescence spectra studies. V3W displays the most dramatic protection followed by L20W and then V31W again in accordance with the suggested extent of interfacial penetration.

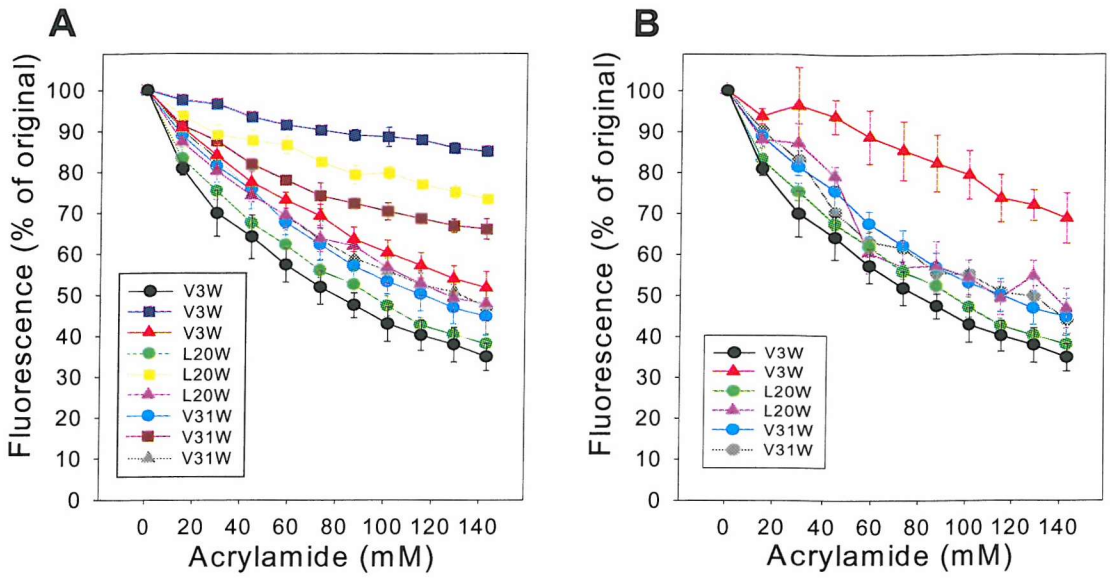


**Figure 4.10 Acrylamide quenching of V3W, L20W and V31W**

Quenching was carried out with 0.2μM protein in buffer 20mM Tris, 1mM EGTA (●), DOPG (■) and DOPC (▲). Excitation was at 290nm and the decrease in fluorescence intensity at the wavelength maxima for each mutant is plotted against amount of acrylamide added. The phospholipid concentrations were 180μM (**A**) and 1mM (**B**). Data shown are means ± S.D. (n=3).

In the presence of 180μM DOPC (figure 4.10A) only V3W displays a significant amount of protection compared with the quenching curves seen in buffer. These results suggest that a small amount of binding occurs with V3W at 180μM DOPC although this was undetectable using the tryptophan emission spectra. It is noteworthy that although the L20W and V31W showed the most significant changes in their spectra in the presence of 180μM DOPG these are not reflected by the quenching data.

When the concentration of DOPC was increased to 1mM for the fluorescence spectra studies, the V3W mutant demonstrated a change approaching that seen with DOPG, and the V31W too showed a significant though less pronounced change. In light of this, quenching with acrylamide was performed in the presence of 1mM DOPC and the results shown in figure 4.10B. V3W shows a significant decrease in its accessibility to acrylamide compared with the values seen when in buffer or 180μM DOPC (figure 4.10A), suggesting that more of the enzyme is being protected by binding to the membrane surface. V31W under these conditions does not show any appreciable change

in accessibility, in contrast to that seen with the spectral studies discussed above. It should be noted that even in the presence of 180μM DOPG when the enzyme might be expected to be fully bound, considerably less protection is seen with the V31W mutant compared to the V3W and L20W. The L20W mutant shows no appreciable change in the presence of 1mM DOPC, in agreement with the fluorescence spectra studies and activity data.

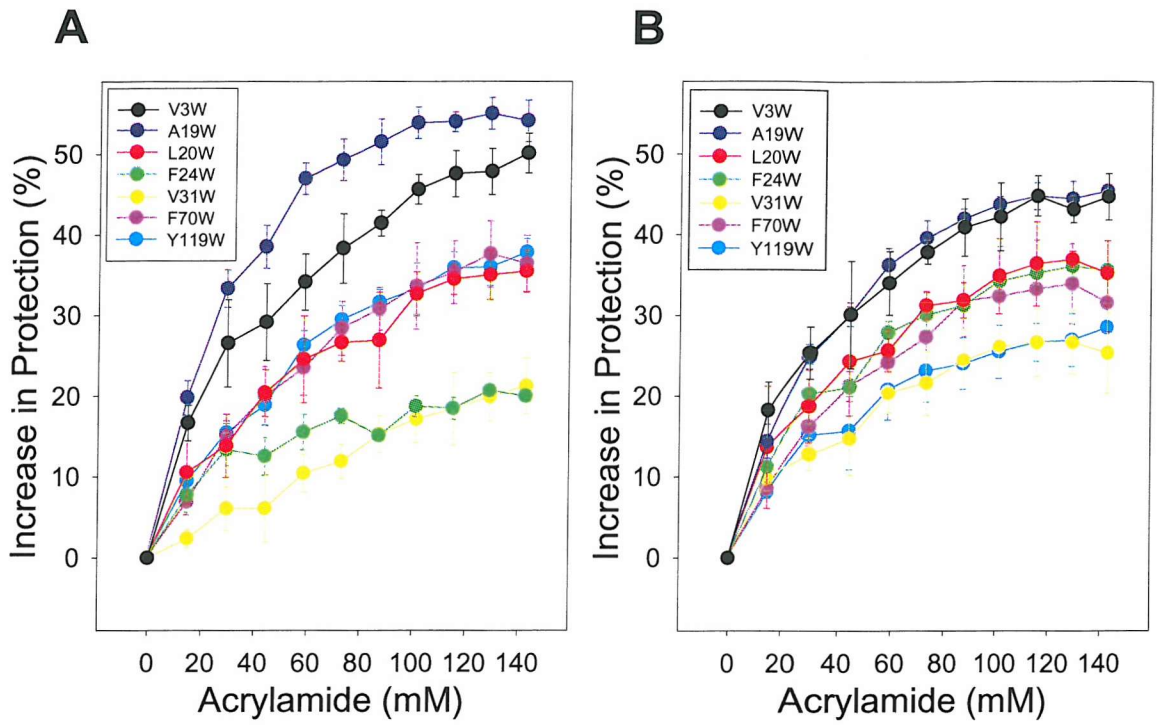


**Figure 4.11** **Acrylamide protection with interfacial binding**

Quenching was carried out with 0.2μM protein in buffer 20mM Tris, 1mM EGTA and in the presence of 100% DOPG (**A**) and 20mole% PG/PC (**B**). Excitation was at 290nm and the difference in fluorescence intensity when bound to vesicles and in buffer at the wavelength maxima for each mutant is plotted against amount of acrylamide added. Data shown are means ± S.D. (n=3).

Quenching studies were then extended to the other PC-inactive interfacial tryptophan mutants in an attempt to gain a qualitative image of the orientation of the hgIIA enzyme when bound to a phospholipid interface. For clarity figure 4.11A shows the increase in relative protection from acrylamide quenching when the enzyme is bound to phospholipid compared to when free in solution. As shown in figure 4.10A the V3W, L20W and V31W mutants showed varying

degrees of protection when bound to 180μM DOPG, a concentration when as discussed previously they would be expected to be fully bound (section 4.2.5). Therefore it was hypothesised that the relative accessibility of the mutants was reporting on the depth of penetration of the protein into the bilayer surface at that position. It can be seen from the results in figure 4.11A that there is a correlation between the position of the tryptophan residue on the protein's surface and the protection seen when binding to a PG interface. The amino terminal residues V3W and A19W show the greatest amount of protection on binding with L20W, F70W and Y119W intermediate, whilst F24W and V31W show comparatively little protection suggesting that they do not penetrate the interfacial surface to a large degree. These results are broadly in agreement with the extent to which the fluorescence emission maxima of the mutants were blue-shifted on binding to DOPG. A model for this binding will be discussed later in section 4.3.

Given the small amount of protection displayed by the V31W mutant and therefore the reduced penetration expected on binding of the protein at this tryptophan residue; the question was raised as to how this mutation could produce the enhancement in binding to PC interfaces suggested by the fluorescence spectral changes seen with 1mM DOPC (figure 4.8F and table 4.4) and the increase in hydrolysis demonstrated with DOPC SUVs (table 4.2). It has previously been demonstrated here (Chapter 3) and elsewhere how the high positive charge on the proteins surface dominates its molecular interactions and physiological role. Due to the anionic nature of the vesicles used here it was very possible that the orientation of the enzyme was determined by the local charge distribution on the interfacial binding surface, particularly on the amino terminal helix implicated in interfacial binding previously (41;70). Quenching experiments with pure PC vesicles were not possible as even at 1mM, significant PC binding was not detected with the majority of the single tryptophan mutants.

It has been demonstrated previously using fluorescence resonance energy transfer that the V3W and L20W mutants bind tightly to 10mole% PG/PC SUVs (data not shown). Consequently quenching was carried out using

20mole% PG/PC of phospholipid to see whether the reduction in charge on the interface would affect how the protein bound. Figure 4.11B shows that with a reduction in charge on the interface there is a change in the amount of protection at each position around the hydrophobic collar. V3W and A19W reduce in the amount of protection they display and become quite similar. L20W and F70W remain relatively constant, whilst F24W shows a dramatic increase in protection and Y119W a dramatic reduction. V31W exhibits a significant though less pronounced increase in protection compared with F24W.

Overall the amount of protection seen around the hydrophobic collar became more similar, suggesting that the protein rotates around an axis between L20W and F70W and that the collar sits more parallel in relation to the plane of the membrane. This result demonstrates that the V31W mutant is probably able to enhance binding and consequently hydrolysis of PC interfaces as it is located closer to the membrane surface under these conditions of reduced electrostatic interactions. It was shown earlier using high salt concentration with PG that under conditions of reduced electrostatic interactions the enhanced interactions of V3W, V31W and V3,31W are discernible and that these mutants were able to hydrolyse the phospholipids under these conditions. The quenching results shown here suggest that the method by which this may occur for V31W is due to the protein being oriented differently to the plane of the membrane and that this orientation is dependent on the charge properties of the interface. This proposal is discussed in more detail in 4.3.

### 4.2.7 Binding to zwitterionic interfaces

The marked differences observed between the various tryptophan mutants and their abilities to hydrolyse DOPC vesicles (table 4.2) were, it was assumed, a direct reflection of their ability to bind to this interface. The results obtained from fluorescence spectra studies and acrylamide quenching have so far shown this contention to be probable. In order to reinforce these

findings binding studies were undertaken using sucrose loaded vesicles according to the method of McClaughlin (15). A series of binding reactions were set up with varying concentrations of non-hydrolysable phospholipid present in the form of sucrose-loaded large unilamellar vesicles. A fixed concentration of enzyme was added to these reaction tubes and the vesicles pelleted by ultra-centrifugation. The enzyme remaining in the supernatant was then assayed using the PpyrPM fluorescence assay described in Materials and Methods (section 2.1.11.3) and the apparent $K_d$s calculated.

Pure PC vesicles can not be pelleted by ultra-centrifugation unless they are doped with an anionic phospholipid, this is assumed to be due to the fact that pure PC vesicles 'leak' (M. Gelb personal communication). Although PC vesicles will pellet with as little as 10mole% anionic phospholipid this level of doping is not very reliable and samples are frequently wasted. Trials were carried out (data not shown) to establish the level of anionic PS to incorporate into the vesicles to ensure reliable pelleting whilst retaining the maximum discrimination between the various mutants in terms of their binding properties. A level of 18mole% of $DO_{Et}PS$ in $DO_{Et}PC$ was selected.

Comparison between the results in figure 4.12, table 4.5 and the activity data in table 4.2, demonstrate quite clearly that there is a direct correlation between the enzymes' ability to bind to these condensed zwitterionic interfaces and their ability to catalyse the hydrolysis of the phospholipids making up this interface.

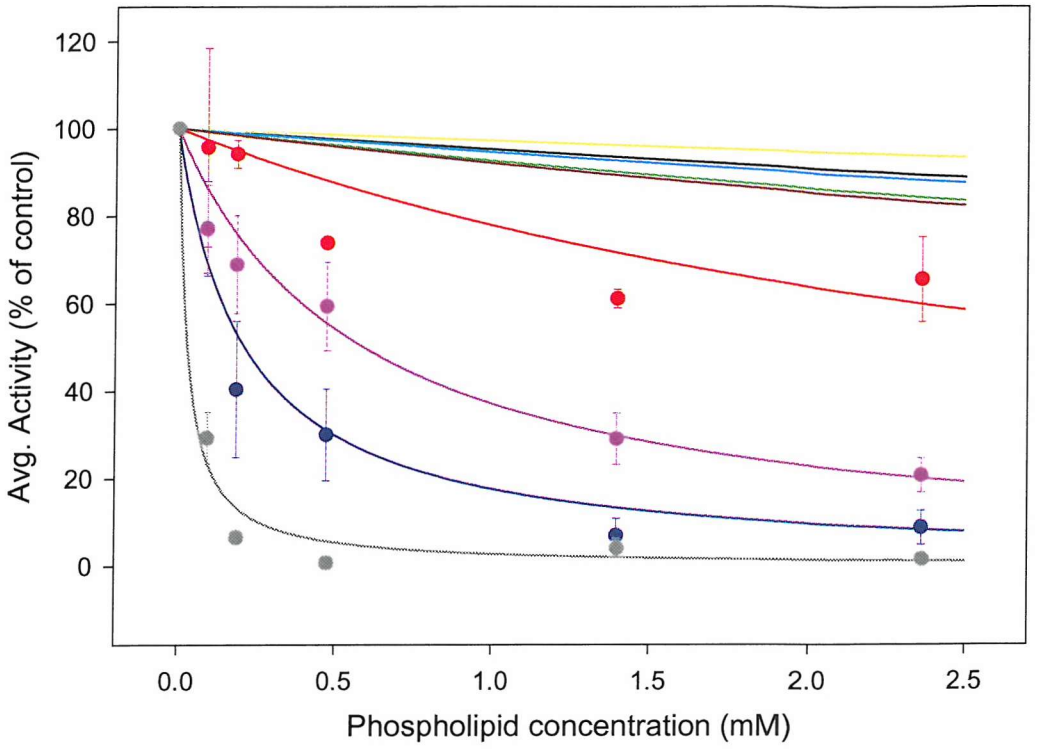


**Figure 4.12 Binding curves of hgIIA sPLA$_2$ and interfacial tryptophan mutants**

The enzymes (0.4μM) were prepared and spun down by ultra-centrifugation in varying concentrations of sucrose loaded vesicles of 18mole% DOEtPS in DOEtPC (see Materials and Methods). The supernatant was then assayed using the PpyrPM fluorescence assay of Radvanyi (76) and the apparent $K_d$s calculated using Sigma Plot™ from mean values ± S.D. (n=3). Wild type – black, V3W – blue, A19W – red, L20W – green, F24W – yellow, V31W – pink, F70W – cyan, Y119W – brown, V3,31W – dark grey. Data points are plotted for mutants displaying detectable binding.

It can be seen that the double mutant V3,31W which demonstrated approximately equivalent activity to the PC active hgV enzyme has an apparent $K_d$ of ~30μM on 18mole% PS/PC LUVs whereas the V3W has a $K_d$ of ~200μM, V31W $K_d$ ~600μM and the A19W an extrapolated $K_d$~4mM with the rest of the tryptophan mutants of the hydrophobic collar like the wild type enzyme showing no appreciable binding under these conditions.

| Binding Data 18mole% PS/PC | | |
|---|---|---|
| | $K_d$ (mM) | S.D. |
| Wild type | 19 | 19 |
| L2W | 10 | 0 |
| V3W | 0.21 | 0.06 |
| A19W | 3.5 | 1.7 |
| L20W | 13 | 2 |
| F24W | 36 | 8 |
| V31W | 0.59 | 0.1 |
| F70W | 18 | 5 |
| Y119W | 12 | 0 |
| V3,31W | 0.029 | 0.004 |

**Table 4.5** **Summary of binding data with sucrose loaded vesicles**

The enzymes (0.4μM) were prepared and spun down by ultra-centrifugation in varying concentrations of sucrose loaded vesicles of 18mole% DOEtPS in DOEtPC. The supernatant was then assayed using the PpyrPM fluorescence assay of Radvanyi (76) and the $K_d$s calculated as detailed in Materials and Methods from mean values ± S.D.

These results confirm the observed changes in tryptophan fluorescence, when the protein is in buffer and the presence of 180μM/1mM PC as well as the quenching data. These results demonstrated that only the V3W of the single mutants was bound to any significant extent at a concentration of 1mM PC. It can be seen from the results presented here that the A19W mutant also exhibits some binding although the values obtained, as can be seen by the error (table 4.5), are on the limit of detectable binding using these assay conditions. A significant increase in binding of the A19W although not detectable as enhanced activity with DOPC vesicles (table 4.2) is consistent with a role for total hydrophobic area in enhancing hydrophobic binding at the interface. The effect of mutagenesis on hydrophobic area has been considered by others (15) and is discussed in section 4.3.

### 4.2.8 Mammalian Cell Membrane Hydrolysis

The ability of human $sPLA_2$s to hydrolyse cell membranes would be expected to be a major factor in determining the physiological roles of these enzymes. These secreted enzymes normally operate in an extra-cellular environment exposed to mM $Ca^{2+}$ concentrations where they would interact with the external monolayer of the plasma membrane and the monolayer coat of lipoproteins. Therefore, an important measure of enzyme activity is that expressed against such physiological phospholipid interfaces. The continuous fluorescence displacement assay used here for measuring the activity of the enzymes has the advantage of using normal phospholipid substrates including whole cell suspensions and lipoproteins. The ability of mammalian cells to be hydrolysed by $sPLA_2$s varies considerable with cell type and method of preparation but the relative activities of $sPLA_2$s on different cell preparations do not appear to change (data not shown).

| | THP-1 Cells | | HEK293 Cells | |
|---|---|---|---|---|
| | % (V3W) | S.D. | % (V3W) | S.D. |
| Wild type | 9.2 | 1.1 | 6.5 | 3.0 |
| V3W | 100[α] | 33 | 100[β] | 24 |
| A19W | 8 | 2.1 | - | - |
| L20W | 16 | 2.6 | 4.4 | 4.5 |
| F24W | 8.5 | 2.2 | - | - |
| V31W | 73 | 5.1 | 60 | 11 |
| F70W | 21.1 | 2.2 | - | - |
| Y119W | 6.8 | 1.4 | - | - |
| V3W,V31W | 182 | 21 | 273 | 34 |
| HgV | 140 | 9.5 | 323 | 49 |
| *Naja naja* | 1085 | 119 | 2113 | 293 |

**Table 4.6** **Relative activities of $sPLA_2$ on mammalian cell membranes**

The absolute specific activity values for $V3W^{\alpha}$ and $V3W^{\beta}$ were 0.5 ± 0.2 and 1 ± 0.2 nmol/min/μg respectively. Specific activities were determined using a fluorescence displacement assay described in (75) against $4x10^5$ cells/ml. Data are means ± S.D. (n=3).

The ability of human $sPLA_2$ and tryptophan mutants to hydrolyse suspensions of THP-1 and HEK293 cells is shown in table 4.6. The activities are expressed as a % of the V3W mutant rather than wild type enzyme because of the inherent difficulty of obtaining accurate values for the negligible activity expressed by the wild type enzyme. The results parallel data using DOPC vesicles and it is clear that the V3W is more effective than the V31W while the remaining single tryptophan mutants do not show a major increase in activity compared to wild type enzyme under these assay conditions. The V3,31W double mutant shows enhanced activity compared with the individual tryptophan mutants consistent with an additive effect on overall activity.

The results with the *N. naja* venom $sPLA_2$ are shown for comparison and highlight the potential of this type of enzyme to hydrolyse cell membranes; this being the physiological role of the enzyme. The expressed specific activity of the venom enzyme is over 10-fold that of the V3W mutant and 6-fold that of the double mutant. Of particular interest is the fact that the V3,31W mutant now expresses very similar activity to the group V enzyme under these physiological assay conditions. Thus it would appear that a double mutation has converted the IIA enzyme into a mutant that could show similar physiological properties to the group V enzyme, especially as both the IIA and V also bind to heparin and to cell surface HSPG.

### 4.2.9 Internalisation of hgIIA $sPLA_2$ into HEK-293 cells

Cho and co-workers have recently shown that the human group V $sPLA_2$ is internalised into unstimulated HEK293 cells in a process requiring both HSPG-binding and tryptophan (W31) dependent plasma membrane hydrolysis (20). They also demonstrated that once internalised the hgV enzyme was targeted to the PC-rich perinuclear membrane by its interfacially active Trp31 and that once localised to this region it released arachidonic acid. In contrast the HSPG-binding but tryptophan-less wild type human group IIA enzyme was not internalised into unstimulated cells. Uptake of wild type hgIIA and the W31A

hgV enzymes was seen into cells where the plasma membrane was hydrolysed by previous treatment with *N. naja* venom $sPLA_2$ or where the cells had been activated by pre-treatment with the cytokine IL-1β (20). Once internalised under these conditions both of these enzymes showed diffuse cytoplasmic localisation and phospholipid hydrolysis. The ability of the V3,31W double mutant to hydrolyse cell membranes and PC vesicles at a very similar rate to the group V enzyme would suggest that this mutant enzyme now possessing both HSPG-binding and cell hydrolysing ability should be internalised into unstimulated HEK293 cells.

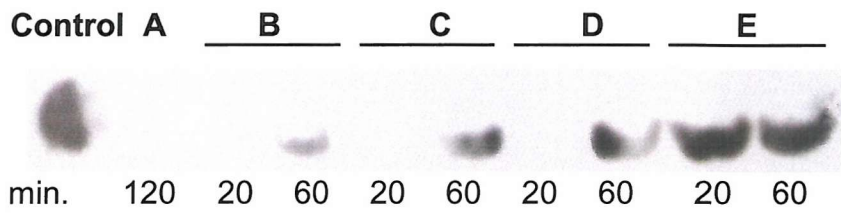


**Figure 4.13** **Comparison of the internalisation of wild type and tryptophan mutants of hgIIA $sPLA_2$ into HEK293 cells detected by Western blotting analysis**

HEK293 cells in DMEM were incubated with 100nM wild type (A), L20W (B), V31W (C), V3W (D) and V3,31W (E) human group IIA $sPLA_2$ for 20 and 60 minutes. The wild type enzyme incubation was extended to 120 minutes. A control lane was run with 20ng of wild type enzyme. Essentially the same results were obtained from duplicate experiments described in Materials and Methods.

HEK293 cells that were freshly grown up from frozen stock samples were incubated with wild type human group IIA and tryptophan mutants for up to 120 min. After washing with medium containing high salt to remove any absorbed enzyme, the cells were isolated, disrupted and the resulting supernatant analysed by SDS-PAGE followed by western blotting following the protocol described in (20). The results are visualised in Figure 4.13. It can be clearly seen that there is a complete failure of the wild type enzyme to be internalised even after incubating with the cells for 120 minutes. In contrast, maximum uptake of the V3,31W double mutant is clearly seen after 20

minutes. Some internalisation of all single mutants is seen after 60 minutes being most apparent with the V3W and just visible with the L20W mutant.

These results correlate with the membrane hydrolysis activity shown in table 4.6 and appear to provide a more sensitive measure of the membrane hydrolysis that is compulsorily required for enzyme uptake. Some internalisation is seen for the L20W mutant although the rate of actual membrane hydrolysis (table 4.6) is indistinguishable from that seen with the wild type enzyme. This probably reflects the much longer time of cell membrane exposure to enzyme with the uptake studies compared with initial rates that are measured in the enzyme assays.

Overall, these results are a remarkable example of a gain in physiological function as a result of mutagenesis. It further emphasises that the lack of ability of the gIIA enzyme to hydrolyse host cell membranes is crucial in terms of the potential physiological functions of this enzyme.

## 4.3 Discussion

Interfacial binding is an absolute requirement for $PLA_2$s acting on physiological phospholipid substrates that contain long chain fatty acids because of the very low monomeric concentration of phospholipid in the aqueous phase (CMC $<10^{-9}$M). Consequently, the enzyme can only access individual substrate molecules and carry out catalysis if it binds productively to the surface of the phospholipid aggregate. Thus interfacial binding can play a very important role in regulating enzyme activity whether inside or outside the cell.

Interfacial discrimination is an important property of the human group IIA $sPLA_2$. This enzyme expresses high activity on anionic interfaces such as those presented by PG but demonstrates negligible activity on the zwitterionic interfaces present as the external monolayer of the plasma membrane or the

monolayer coat of lipoproteins. Such discrimination is compatible with the anti-microbial activity of this enzyme in that the bacterial membrane is highly anionic being rich in PG and this allows high affinity binding and phospholipid hydrolysis resulting in bacterial killing (77). The extra-cellular level of the IIA enzyme can rise over 1000-fold under conditions of acute infection while such high concentrations (~10μg/ml) are normally present in human tears (58) and semen (119). Therefore, the inability of this enzyme to hydrolyse the normal host cell membrane or lipoprotein is very important. The enzyme is now regarded as an acute phase protein (61).

Many studies of protein-membrane interactions have now highlighted a major role for tryptophan in these interactions as this residue has a propensity to partition into the interfacial region of the phospholipid bilayer (or monolayer) of PC vesicles (section 4.1 (11)). Tryptophan also plays an important role in positioning α-helical regions of integral membrane proteins within the bilayer (11;103;106). Such partitioning will facilitate the interfacial binding to condensed PC interfaces of peripheral enzymes that contain tryptophan on their interfacial binding surface, and this has been reviewed in the case of the $sPLA_2$s (12). Moreover, such a preference for PC interfaces may provide a membrane targeting mechanism for proteins within the mammalian cell to the PC-rich perinuclear membrane (114;115) where arachidonic acid release is linked to the production of leukotrienes and prostaglandins potent mediators of the inflammatory response.

The group IIA enzyme does not contain tryptophan and this is consistent with the fact that the enzyme is inactive against zwitterionic mammalian cell membranes. If tryptophan residues, as suggested, play a significant role in defining $PLA_2$ function, then the tryptophan profiles of other $PLA_2$s together with other aromatic residues in the interfacial region may be crucial to understanding function. Moreover tryptophan insertion or removal by mutagenesis could change the physiological properties of the particular phospholipase. In determining the position of tryptophan insertion into the interfacial binding surface of the human group IIA enzyme, the location of

interfacial tryptophan residues in other related $sPLA_2$s with different physiological properties provides an important template.

Part of the aim of this chapter was to demonstrate how tryptophan insertion can enhance the PC activity of hgIIA and to investigate the feasibility of changing the characteristics of the human IIA $sPLA_2$ to that of the human group V enzyme by tryptophan insertion mutagenesis. This human group V enzyme contains two conserved surface tryptophans at positions 31 and 79, one of which (W31) is on the putative interfacial surface. Mutation of this residue (W31A) but not W79A resulted in an enzyme with 40-fold reduced activity with DMPC vesicles and also reduced activity on cell membranes (46). Thus the presence of tryptophan in the group V enzyme does allow this extracellular enzyme to hydrolyse cell membrane phospholipid, which in the case of neutrophils parallels the release of leukotriene B4 by these cells (46). In addition the group V enzyme, like the group IIA enzyme, binds to HSPG and this appears to be an important characteristic in terms of physiological function (93;112;113;120). Thus the equivalent V31 position of the IIA enzyme was an obvious site for mutagenesis, as was comparison with the V3W mutant of the IIA enzyme where greatly enhanced activity on zwitterionic interfaces has already been described (48).

The unusual properties of the human IIA $PLA_2$ in that it is virtually inactive against PC vesicles and cell membranes provides a powerful system for studying catalytic *enhancement* as a result of mutagenesis. The production of a mutant with relatively low absolute specific activity will be reflected in a dramatic enhancement of activity when compared to the inactive wild type enzyme. Moreover such enhancement of activity, as opposed to inhibition, is unlikely to be the result of a change in overall protein structure resulting from the mutagenesis.

The results described in this chapter highlight that the insertion of a tryptophan residue at position 31 (V31W) results in a significant (20-fold) enhancement of activity against PC vesicles. The ability of the V3W mutant to enhance such activity over 30-fold under these conditions means that this mutant remains

the most effective. Of particular note is the fact that the double mutant, V3,31W is over 60-fold more active than the native enzyme indicating that the two effects are additive. The mutation of all the other non-polar residues in the hydrophobic interfacial collar of the IIA enzyme to tryptophan failed to produce the dramatic rate enhancements seen with the V3W and V31W mutants. It is probable that factors such as residue exposure, orientation and an increase in exposed hydrophobic surface area as a result of the mutagenesis to tryptophan (15) are important in producing enhanced binding to zwitterionic interfaces. The increased binding of V3W and V31W mutants to DOPC has been demonstrated in line with the enhanced rates of hydrolysis. Moreover, the binding of the V3,31W double mutant to DOPC (180μM) vesicles was directly detected as a result of a small shift in the wavelength emission maximum.

A recent study on the role of ionic, aliphatic and aromatic residues on membrane-protein interactions using SPR (101) highlight the potential role of electrostatic interactions in bringing the enzyme in close contact with the membrane interface. Subsequent membrane penetration and resulting hydrophobic interactions involving aliphatic and aromatic residues stabilises the membrane-protein complex. The overall $K_d$ for a PC interface determined by SPR was about 0.1μM, a value that should have resulted in substantial binding of the group IIA enzyme under these assay conditions. An explanation for the discrepancy is that SPR detects a non-specific encounter complex whereas catalysis and changes in tryptophan fluorescence require a subsequent and specific membrane penetration event involving hydrophobic interactions.

Quenching studies with the three tryptophans representative of those found in other $sPLA_2$s supported the binding data from enhanced hydrolysis of PC and fluorescence spectra and showed that V3W bound most tightly followed by V31W and that under these conditions minimal binding was observed for the L20W mutant.

Cell membrane hydrolysis results with both THP-1 and HEK293 cells correlate well with the PC vesicle data demonstrating that only the V3W, V31W and V3,31W double mutant display enhancement on physiological neutral interfaces. The comparison of the ability of the human group IIA and group V enzymes to hydrolyse cell membranes highlights that in the case of the V3,31W double mutant, this enzyme shows very similar catalytic properties to the group V enzyme. The other important property of both enzymes is their ability to bind to cell surface HSPG. The linkage of such binding to enzyme internalisation has been demonstrated in the case of the IIA enzyme (94,112) and most recently in the case of the group V enzyme (113).

In the case of the group IIA enzyme cell transfection studies implicated the enzyme in the delayed prostanoid response where the enzyme was internalised in a glypican-linked event following $cPLA_2$ activation (95). Glypican is a GPI-anchored HSPG and binding to this molecule was linked to internalisation and translocation to the perinuclear compartment in proximity to COX-2 (reviewed in (94). Notably it was not possible to mimic this overall process by the exogenous presentation of the group IIA enzyme (95), an observation that casts serious doubt on the interpretation of data involving transfected cells that over-express the enzyme.

In the case of the group V enzyme, two distinct scenarios have emerged depending on the type of cell being investigated. The internalisation of externally added hgV enzyme into unstimulated HEK293 cells was linked with both HSPG-binding and the ability to hydrolyse cell membrane phospholipid (20). Once internalised the enzyme was targeted to the perinuclear region where phospholipid hydrolysis occurred and was associated with enhanced eicosanoid synthesis. In addition to this the enzyme also led to the recruitment of 5-lipoxygenase to the nuclear envelope where the two enzymes co-localised (20). In contrast to HEK293 cells, the uptake of the hgV enzyme by human neutrophils (113) leads to protein degradation and it has recently been argued that hgV enzyme carries out its physiological effect on the external leaflet of the plasma membrane in the case of neutrophils. External cell membrane hydrolysis and the release of free fatty acids and lyso-PC have

been suggested to lead to the rise of intracellular $Ca^{2+}$ leading to the translocation of $cPLA_2$ and the lipooxygenases to the perinuclear membrane while phosphorylation leads to the activation of $cPLA_2$ (113). Leukotriene biosynthesis would then further activate the neutrophil in a positive feedback response.

If the physiological effects on cells of the group V enzyme are simply a reflection of its plasma membrane-hydrolysing and HSPG-binding properties then it was hypothesised that the V3,31W-double mutant of the IIA enzyme should have the same physiological effects. The results presented here have been able to show that unlike the wild type IIA enzyme, the double mutant is able to hydrolyse the cell membranes and is internalised into unstimulated HEK293 cells consistent with this hypothesis. Work from the Cho laboratory (20) showed that HEK293 could be activated by prior treatment with IL-1β. Such cells became in some way sensitised to the action of the group IIA enzyme, which was now internalised. Interestingly the IIA enzyme showed a diffuse cytoplasmic signal for membrane phospholipid hydrolysis compared to the perinuclear hydrolysis after uptake of the group V enzyme (20). It would be anticipated that the presence of tryptophan residues in the double mutant should target this enzyme to the perinuclear membranes of the cells and promote arachidonic release together with prostaglandin synthesis (95;112). The role of tryptophan residues in targeting proteins to the PC-rich perinuclear membrane has been discussed elsewhere (95;112).

Overall, our understanding of the molecular interactions that govern interfacial binding and activation is not complete, nor is the topological relationship between the protein and the interface (see later). This study highlights the fact that certain hydrophobic residues within the interfacial-binding surface facilitate productive binding to zwitterionic interfaces when converted to tryptophan. The ability to enhance the activity of the human group IIA enzyme by minimal (single or double) mutations suggests in evolutionary terms that the selective hydrolytic properties of the IIA enzyme have evolved to reflect physiological function. By the same argument the properties of other mammalian enzymes such as the group V must reflect a different

physiological role for the enzyme. In both cases it is the interfacial binding step (and other surface properties of the enzyme) that must be dictating physiological function. The group V enzyme shows modest antimicrobial activity *in vitro* (23;87;121) consistent with the surface properties of this cationic HSPG-binding protein. However, it is not clear that such activity is physiological relevant compared to the IIA enzyme where serum concentration of this acute phase protein can exceed 10μg/ml under conditions of acute infection.

The increases in activity seen on PC interfaces has been confirmed by binding studies with sucrose loaded vesicles to be directly correlated to the binding affinity of the tryptophan mutant, as the double mutant V3,31W showed considerably enhanced activity and the highest binding affinity followed by the V3W and V31W mutants. Not all the positions mutated were sensitive to tryptophan substitution and this is possibly indicative of how the protein sits on the membrane or a reflection of the relative change in exposed hydrophobic surface area. Relatively large hydrophobic residues frequently take the positions insensitive to tryptophan insertion in the native protein, or where small residues are present where they line the entrance to the active site slot. There is no increase in activity with PC interfaces for the A19W mutation despite an enhancement in binding observed with sucrose-loaded vesicles. This is possibly a reflection of the residue's position as A19W is situated on the wall of the active site slot. Human group IIA has a 3 to 4-fold lower preference for PC as opposed to PG headgroups, reflecting the enzyme's small active site entrance. The further reduction in the size of this entrance by the insertion of a bulky tryptophan in the place of a small alanine may make PC hydrolysis even less favourable.

It has been shown that the fluorescence emission maximum is a reliable marker for the environment of a tryptophan residue in a protein, including its relative penetration into a membrane environment (103;122). Attempts to use the emission maximum to assign membrane insertion depths have been hindered by sequence effects (107;123;124). Under these conditions the

wavelength emission maximum would be affected by quenching effects due to a complicated balance of new and enhanced quenching and the reduction of internal static quenching by both local amino acid residues and interfacial effects. Despite these complicating factors it is tempting to speculate from the blue-shifted wavelength emission maximum results that the hgIIA when bound to an interface penetrates more deeply at the amino terminal helices 1 and 2 and that loop 2 containing residue V31W is relatively shallow in its penetration of the bilayer.

This assertion is supported by the quenching studies performed with all seven viable tryptophan mutants of the hydrophobic collar. Acrylamide as discussed previously is an excellent collisional quencher of tryptophan fluorescence and yet has limited access to phospholipid interfaces. Although the interfacial region of the bilayer is relatively hydrated there exists a concentration gradient of water and hence acrylamide quenching should be able to give an indication of the depth of penetration of a tryptophan residue into the bilayer. The acrylamide protection data obtained supports this contention and the molecular basis for the wavelength emission maxima discussed above. Acrylamide quenching used in this way can only provide a qualitative orientation for the protein relative to the bilayer surface. Despite this limitation, a model is proposed (figure 4.14A) where the enzyme sits on the interface with no penetration passed the glycerol backbone of the phospholipids as indicated by both the fluorescence spectra shifts (331nm is the most blue-shifted) and quenching data (all the tryptophans are quenched if only to a limited extent when bound to the interface). The amino terminal helices 1 and 2 penetrate most deeply into the interfacial region of the bilayer and loop 2 containing V31W is most exposed to the aqueous environment suggesting that the putative interfacial binding surface, as it is classically viewed, is positioned at an angle to the plane of the interface.

The model shown in figure 4.14A has been supported by Gelb and co-workers (125), in their recent study with hgIIA. In that study they used the same quantitative method of EPR and spin labelling as used to elucidate the binding of the bee venom enzyme to an interface (section 1 (18)). Because of

technical aspects of their methodology it was necessary for them to use high concentrations of $sPLA_2$ (~50μM) and phospholipid (25mM). Under these conditions they found that hgIIA induced aggregation of anionic LUVs via clustering on the membrane surface and subsequent cross-linking of the vesicles to form a supra-molecular aggregate and they suggested that this explained a number of anomalous observations made for this protein regarding its binding to anionic vesicles.

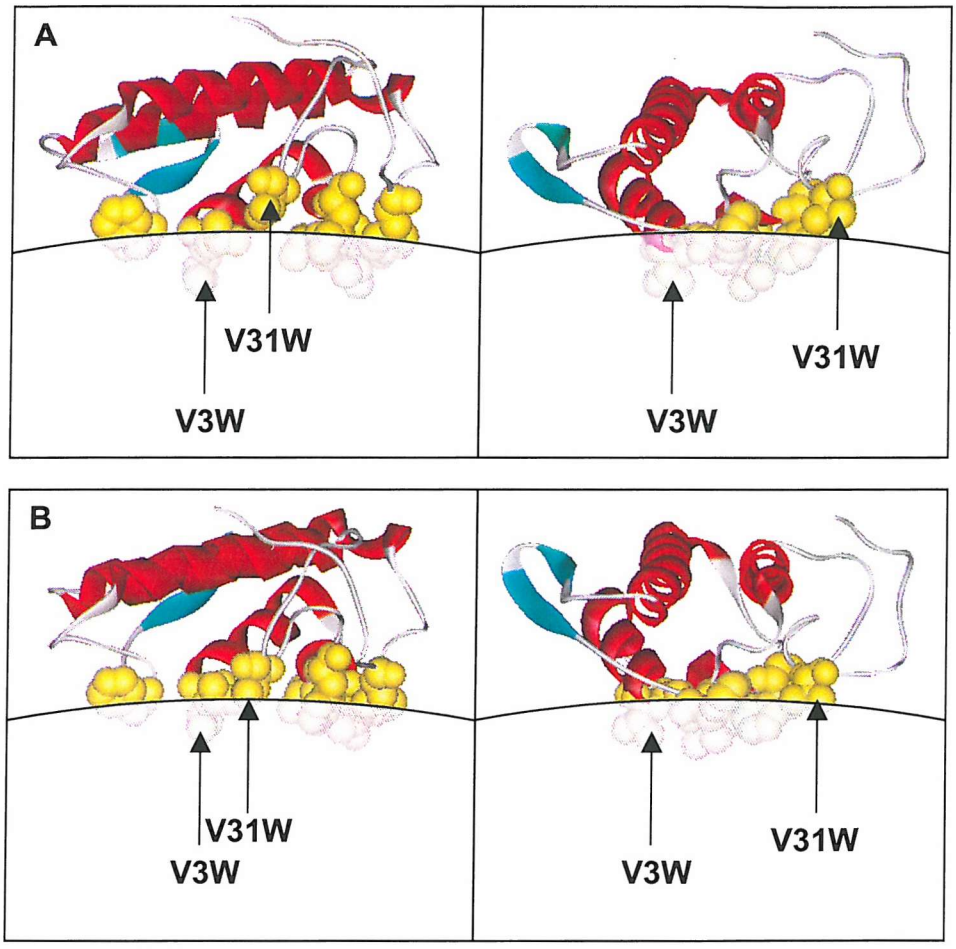


**Figure 4.14** **Model of hgIIA binding to A) 100%PG and B) 20mole% PG/PC small unilamellar vesicles using quenching data**

Ribbon structure of hgIIA bound to SUVs. Non-polar residues of the hydrophobic collar mutated to tryptophan are shown in yellow. The surface of the SUV is represented by a curved line and is drawn to scale. **A)** represents the orientation with 100%PG and **B)** represents the orientation with 20mole%PG/PC. The figures on the right are a 90° anti-clockwise rotation of the left-hand figures

In order to circumvent this problem they used mixed micelles, which have a diameter of ~10nm, so that hgIIA which they suggested was comparatively similar in size (approximately half) was not able to form clusters on the same micelle and hence aggregation would not occur. Under these conditions they reported a binding model (figure 4.15A) very similar to the one proposed in this thesis. The conditions used here with SUVs might be expected to cause similar aggregations as detected by Gelb and co-workers and the results presented here would suggest that even under possible aggregating conditions hgIIA is able to bind to the interface in a similar manner to when bound to the interface in monomeric form.

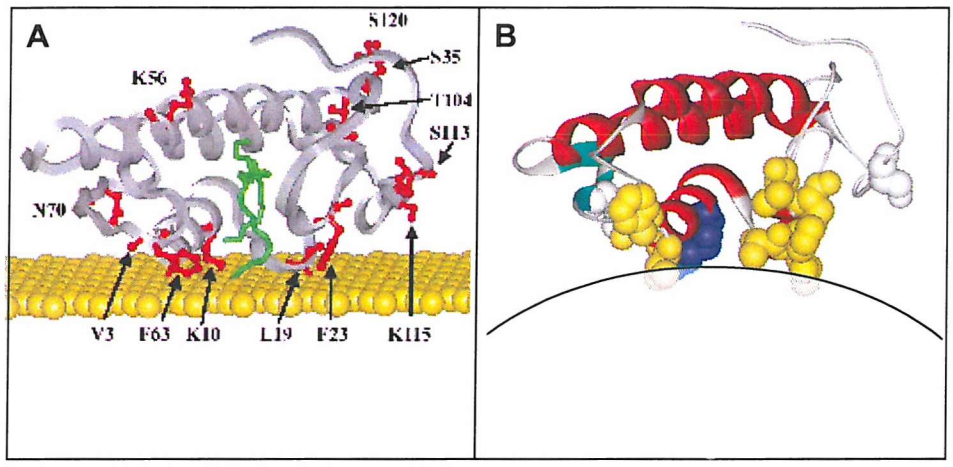


**Figure 4.15** **Model of hgIIA binding to mixed-micelles using spin-relaxant data**

**A)** Ribbon structure of hgIIA bound to mixed-micelles taken from (125) with permission. The surface of the mixed-micelle is represented as a planar surface with yellow balls (radius 2.5Å) and a phospholipid analogue is shown bound to the active site in green. **B)** The same orientation based on **A**) with the surface of the mixed-micelle represented by a curved line and drawn to scale. Non-polar residues of the hydrophobic collar mutated to tryptophan in this study are shown in yellow for comparison to figure 4.14A.

Despite the similarities between the two models there are aspects of the EPR work that raise concerns. Firstly, given the highly curved surface of the mixed micelle (figure 4.15B) it seems strange that a lysine residue distal to the IBS is completely protected from aqueous relaxant. Secondly, in figure 4.15A the binding model that is proposed is represented on a planar surface when the data was obtained from binding to a highly curved surface as depicted in

figure 4.15B. These disparities and the data presented here suggest that the hgIIA enzyme on binding to an interface may induce conformational changes to the membrane surface or the protein itself may undergo conformational changes. The enzyme might be imagined to create an indentation in the bilayer surface sufficient to explain the blue-shifts in wavelength emission maximum and acrylamide protection seen with the Y119W mutant situated on the edge of the interfacial binding surface and the protection from aqueous relaxant of the spin label at position of K115 in the spin label investigation. Alternatively it may be possible, though perhaps less likely due to the 7 disulphide bonds present in this small compact protein (figure 1.4), that the enzyme itself undergoes conformational changes to bring more distal parts of the interfacial binding surface such as Lys115 and Tyr119 in contact with the membrane. Such required changes whether in protein or membrane would be in line with the reduced activity of the wild type enzyme on anionic interfaces when compared to the charge reversal mutants in Chapter 3, sections 3.2.3,3.2.4 and 3.2.7, and with the tryptophan mutants in the presence of high salt in this chapter. It appears that the high positive charge on the protein causes it to bind to the interface in a manner that results in a catalytically less productive conformation when compared to situations of reduced electrostatic interactions.

Both the quenching data presented here and the recent EPR study (125) raised the question of how the V31W mutant of hgIIA, positioned as it seems to be away from the IBS itself, is able to interact with zwitterionic interfaces and enhance hydrophobic interactions with anionic interfaces in the presence of high salt. The model presented in figure 4.14B shows the suggested orientation of the enzyme when bound to a 20mole% PG/PC vesicle where the electrostatic contributions from the IBS residues will be markedly reduced. Under these conditions the hydrophobic collar of the IBS might be able to exert a more pronounced effect on the orientation of the enzyme and consequently sit more parallel with regard to the plane of the membrane. The results shown here suggest that the enzyme is rotated through approximately $15^{o}$ about an axis between residues L20W and F70W bringing the V31W residue closer to the interface where the enhancing effect of the tryptophan

residue can take effect. Altered binding has been noted before with charge reversal mutants of hgIIA where R7E/K10E/K16E mutant was seen to bind in a different orientation to the wild type protein (41) and with the *Naja naja* group IB enzyme which binds to zwitterionic interfaces ~3-fold more tightly than to PG and has substantially different phospholipid footprint and $K_d$ values (13).

Very recent binding studies by Gelb and co-workers carried out using mutants of the hgIIA and hgX enzymes have shown that the basic residues of the IIA at positions 7 and 10 as opposed to the glycine residues present at these positions in hgX are not the critical factors in preventing the hgIIA from binding to PC interfaces and permitting binding of the group X enzyme (108). When they produced a V3W mutant of the hgIIA, as used here, they noted that it caused a 90-fold increase in binding affinity of the enzyme for 20%PS/PC vesicles. The binding affinity of the IIA enzyme actually exceeded the group X under these conditions. Mutating the interfacial tryptophan W67 of the group X enzyme to an alanine led to an 8-fold reduction in binding to the same vesicles and an 18-fold reduction to pure PC vesicles with no change in anionic vesicle affinity. Importantly when added exogenously to cells they found that the W67A mutant of hgX had reduced ability to release arachidonic acid from those cells.

They also demonstrated that the triple mutant V3W/R7G/K10G hgIIA mutant showed a 6-fold increase in PC affinity over the V3W mutant. They suggested that the lag in phospholipid hydrolysis seen with the group IIA enzyme under these conditions using PC substrate is due to the lack of affinity for these interfaces and that as the products from hydrolysis accumulated so the enzyme's activity increased. This lag characteristic is well known feature of the pancreatic IB enzyme. They suggested that this increase in activity was due to both more enzyme being recruited to the membrane surface and importantly through increased interfacial activation. Notably, they proposed that interfacial binding itself was not sufficient to cause interfacial activation with hgIIA on PC interfaces, as the V3W hgIIA enzyme bound 10-fold more tightly to PC-rich vesicles than the wild type hgX yet still retained a lag in hydrolysis and was 40-fold less active on PC interfaces than the group X (108).

The studies of the binding properties of $sPLA_2$ by Gelb and co-workers were extended to encompass the full repertoire of known mouse and human enzymes (109). The results presented by them demonstrate that all had similar substrate preference and this supports the assertion that the different physiological role of the enzymes must be a product of the enzymes' interfacial specificity. It was demonstrated that all the enzymes with an interfacial tryptophan with exception of the group IBs showed no lag with PC hydrolysis and were able to release arachidonic acid when added to mammalian cells. In contrast enzymes with no interfacial tryptophan (the mouse and human groups IIA, IIE and IIF) had low binding affinity to PC and demonstrated an appreciable lag on hydrolysis. They also demonstrated that the over-expression of PC inactive enzymes in transfected cells led to release of arachidonic acid suggesting that the $sPLA_2$ in this context hydrolyse other membranes distinct from the plasma membrane where exogenously present $sPLA_2$ would be expected to act. These results may explain earlier results implicating the group IIA enzyme in the production of mediators of the inflammatory response (94;95).

Taken together these two studies support the evidence presented here of the prominent role that tryptophan plays in a $sPLA_2$'s ability to bind to and consequently hydrolyse zwitterionic interfaces. The dramatically different binding and hydrolytic profiles of the various human and mouse enzymes together with varying tissue distribution once more argues strongly for different physiological functions for these $sPLA_2$ and that the interfacial specificity of these otherwise similar enzymes is a prime factor in determining that catalytic and hence physiological function (109).

For future work it remains to be established using fluorescence microscopy if the internalised V3,31W double mutant of the IIA enzyme targets the PC-rich perinuclear membrane region of HEK293 cells as a result of the presence of interfacial tryptophan residues and that such targeting is linked to arachidonic release and prostaglandin synthesis. Presumably the local concentration of $Ca^{2+}$ in the perinuclear region will be high enough to allow hydrolysis as is proposed in the case of the group V enzyme (20). In contrast to HEK 293

cells, a different physiological response would be predicted for human neutrophils where the exogenously added group V enzyme acts on the outer leaflet of the cell to release fatty acids and is internalised in order to terminate this action and degrade the enzyme (126).

The results shown here demonstrate that the hgIIA $sPLA_2$s lack of activity on such phosphatidylcholine interfaces can be overcome by the introduction of tryptophan residues at two positions on the proteins interfacial binding surface V3W and V31W. At this time there is insufficient evidence to state whether this enhancement is specific for tryptophan residues or whether it simply reflects the relative size increase of the exposed hydrophobic residue at these positions. These two positions on the enzyme's surface, which led to enhancement of zwitterionic hydrolysis, are taken by the same relatively small hydrophobic residue (valine) in contrast to the other interfacial mutants, which showed no enhancement in activity. The only other interfacial mutation to affect binding to PC interfaces was the mutation of another small residue, when the Ala19 was mutated to Trp, again suggesting that the increase in relative hydrophobic surface area is important to the enhanced binding of the protein. However the very small influence of what is a large change in hydrophobic area (ala→trp) highlights the importance of exposure of the particular residue on the surface of the protein. Alanine 19 has a relatively buried position on the interfacial surface.

Further work regarding the importance of hydrophobic area may be undertaken using V3L and V31L mutations. If the enhancement of the PC activity is linked to exposed hydrophobic surface area as opposed to specific properties of tryptophan then the introduction of the relatively large but unaromatic Leu residue should lead to significant catalytic enhancement.

# Chapter 5 – General Discussion

## 5.1 General Discussion and Future Work

The diversity of human $sPLA_2$s being revealed and the range of their tissue distributions are making the determination of precise physiological roles for members of this large family of enzymes very difficult. The high degree of structural similarity shared by the various $sPLA_2$s identified and the lack of major differences in substrate specificity argues for the different physiological roles of these enzymes being dependent upon their interfacial specificity and other surface properties. The hydrolytic activity of $sPLA_2$ on physiological substrates containing long chain fatty acids is dependent on interfacial binding and consequently the binding properties of the various $sPLA_2$ identified is the subject of considerable interest.

The human group IIA $sPLA_2$, the most extensively studied of these enzymes and the subject of this thesis, has emerged as an acute phase protein with potent antibacterial properties against Gram-positive bacteria as a result of membrane hydrolysis. In contrast the enzyme possesses little or no activity on host membranes and serum lipoproteins under normal conditions. The surface of this enzyme is dominated by two properties, a high global positive charge due to the presence of a high number of cationic residues and a lack of interfacial tryptophan residues. Both of these characteristics are entirely consistent with a major role for this enzyme as a selective antimicrobial protein.

Cationic $sPLA_2$s have previously been demonstrated to have antimicrobial activity that was not present with less positively charged or anionic enzymes, on both permeabilised Gram-negative bacteria (54;127) and more recently intact Gram-positive bacteria (86;128). The molecular basis of this charge requirement in $sPLA_2$ mediated antibacterial activity was unproven. Furthermore, it was not known if it was the overall global charge on the protein that was important or if there were specific cationic residues that were required for the antibacterial activity.

It has been demonstrated in Chapter 3 that the potent antibacterial properties of this enzyme, compared with other $sPLA_2$s, is due to its high isoelectric point, making it highly charged under physiological conditions and therefore ideally suited to crossing the anionic outer cell wall of Gram positive bacteria. Indeed using charge reversal mutants of this enzyme its ability to hydrolyse Gram-positive bacterial membranes and consequently exhibit antibacterial activity directly paralleled the loss in overall charge on the proteins surface. These results demonstrated that, as found for heparin binding by the hgIIA, it is the overall charge on the protein that is required for its potent Gram-positive antibacterial activity rather than single residues or clusters of residues.

Initial findings have been presented here on the possible importance of this high charge in the enzyme's potential activity against Gram-negative bacteria. When these usually $sPLA_2$ resistant bacteria are exposed to the outer cell membrane permeabilising agent polymyxin-B-sulphate, human group IIA with its high pI was able to hydrolyse the cell membrane. As with Gram-positive bacteria, this hydrolysing-ability was linked to the overall positive charge on the enzyme surface. The requirement for charge on Gram-negative hydrolytic activity is less pronounced than for the Gram-positive bacteria and probably reflects the differences in the thickness and amount of cross-linking in the peptidoglycan cell walls of these bacteria. This work will need to be expanded upon in order to make a definitive statement regarding the importance of hgIIA's high charge on Gram-negative antibacterial activity and it would be beneficial to repeat the experiments presented here with various Gram-negative bacteria under similar conditions.

The killing of Gram-negative bacteria such as *E.coli* by this human $sPLA_2$ is an excellent example of the need for synergism in the innate immune response that involves a large number of proteins and peptides. Only if the outer membrane of *E.coli* is permeabilised by other agents such as BPI can the enzyme access the bacterial cell membrane. Similarly the antibacterial protein lysozyme can permeabilise the cell wall of many Gram-positive bacteria again facilitating access of the hgIIA enzyme to the cell membrane. It should be noted that most if not all the antibacterial proteins of the innate immune

response tend to be cationic, thus capitalising on the highly anionic nature of bacteria, be it the outer membrane, cell wall or the cell membrane which is enriched in anionic phospholipids such as PG and cardiolipin.

Although an effective antibacterial protein it may be possible to enhance the antibacterial properties of this highly effective enzyme. Human group IIA has greater substrate specificity compared with the porcine pancreatic group IB enzyme and, in particular, the human enzyme expresses very low activity against the anionic bacterial phospholipid cardiolipin, whereas the pancreatic enzyme will rapidly hydrolyse cardiolipin (129). The most likely explanation for this reduced activity with the hgIIA enzyme is a narrower hydrophobic channel leading to the catalytic site which restricts the access of the bulky cardiolipin molecule (129). A major factor producing this narrow channel is His6 on the amino-terminal helix (39). It is hypothesised that mutating His6 of the IIA enzyme to a smaller and more flexible residue such as asparagine (H6N) or alanine (H6A) would increase the size of the hydrophobic channel and allow access of cardiolipin to the active site permitting hydrolysis. Cardiolipin is a major constituent of many Gram-positive bacterial membranes and is second only to PG in proportion of the total phospholipid. The ability to hydrolyse this important phospholipid would be expected to be accompanied by an increase in antibacterial potency.

Another mutagenic strategy would be to enhance the overall catalytic activity of the enzyme. This strategy probably has less potential when anionic membranes are the substrate because the enzyme already has a very high affinity for such interfaces. In fact although tryptophan mutations dramatically enhance the catalytic activity on PC-interfaces it has been shown that they had no effect on anionic interfaces under physiological conditions. This lack of enhanced activity is supported by the observation that the V3W, V31W and V3,31W mutants showed the same activity on Gram-positive bacteria (*M.luteus*) as the wild type enzyme (data not shown).

In the second part of this thesis the lack of activity of the hgIIA on zwitterionic interfaces such as those on the outer leaflet of the plasma membrane and the

monolayer coat of serum lipoprotein has been investigated. Under conditions of acute infection the extra-cellular levels of this protein can rise by over 1000 fold and such high concentrations (~10μg/ml) are normally present in human tears and semen. If the enzyme is to exhibit selectivity for invading bacteria when the concentrations of this protein rise to such high levels the importance of the inability of the enzyme to hydrolyse host membranes and lipoproteins becomes self evident.

Chapter 4 demonstrates the sensitivity of the human group IIA $sPLA_2$ to the introduction of tryptophan residues on its interfacial binding surface. The effect of these insertions was selective and some insertions led the enzyme to have appreciable activity on PC interfaces. Previous work in this laboratory established the ability of a single tryptophan insertion (V3W) to enhance the hydrolysing ability of this enzyme on PC interfaces. This early work has been expanded on here to show that another position on the interfacial binding surface (V31W) is able to cause a major, though less pronounced, enhancement in activity. Consequently, a V3,31W double tryptophan mutant was produced which displayed an additive effect for the inserted tryptophans on PC hydrolysis and comparable activity on mammalian cell membranes to the human group V enzyme.

It is not at this time clear whether the enhancing effect of tryptophan on neutral interfaces represents a unique property of tryptophan residues or whether it is in part a reflection of the size of the exposed hydrophobic residue at key points where the protein interacts with the membrane. In order to ascertain this, the most direct approach is to produce non-polar mutations of equivalent overall size in terms of hydrophobic surface area compared with tryptophan but lacking aromaticity. Therefore, the production and analysis of mutants such as V3L and V31L should be revealing. Such mutants may be equally active as the V3W and V31W mutants confirming the importance of exposed hydrophobic area.

Alternatively, studies may be undertaken with mutant hgIIA containing cysteine residues at equivalent positions to the tryptophans presented here.

The cysteine mutants will be amenable to chemical modification and this would permit other aromatic and hydrophobic molecules to be attached to the proteins to determine the properties which may best represent those required to activate the protein on PC interfaces. Examples already exist of chemical modifications that enhance $sPLA_2$ activity on cell membrane and zwitterionic interfaces; early work on the pancreatic enzyme was performed where acyl groups were attached to specific lysine residues producing an enzyme with enhanced membrane penetrating ability and hydrolytic activity (130).

Internalisation of the hgV enzyme has been implicated in its suggested role in the generation of inflammatory mediators and has been shown to be dependent on one of the enzyme's tryptophan residues and also on its heparin binding capacity, both of which properties the V3,31W double mutant possesses. It was hypothesised that if the physiological effects of the hgV enzyme were simply a reflection of these properties then the V3,31W should be internalised into unstimulated HEK293 cells as was seen with the hgV enzyme. This was indeed found to be the case, in contrast to the wild type hgIIA protein that displayed no detectable internalisation under the same conditions. The hgV enzyme has been shown once internalised to be targeted to the PC rich perinuclear membrane leading to the release of arachidonic acid and prostaglandin synthesis and these effects were found to be dependant on the tryptophan residue W31.

Future work remains to be carried out using fluorescence microscopy to see if the V3,31W mutant once internalised will also display this perinuclear targeting and inflammatory mediator production, in contrast to the wild type protein which would only internalise into HEK293 cells when stimulated with IL-1β and then demonstrated a diffuse cytoplasmic signal and phospholipid hydrolysis. The studies on the internalisation of the group V enzyme provided strong evidence that the W31A mutant of this enzyme, which has greatly reduced capacity to hydrolyse cell membranes, failed to internalise into normal HEK293 cells. These authors then used snake venom $sPLA_2$ to pre-treat the cells and produce membrane hydrolysis, followed by addition of the W31A mutant. Under these conditions the W31A was internalised but not the snake

venom enzyme which does not bind heparin. It would be interesting to see if snake venom treatment would also permit internalisation of the wild type group IIA where a diffuse cytosolic distribution would be expected. This protocol is preferred to IL-1β treatment of HEK293 cells, which produces a very variable response (personal communication from the Cho laboratory).

Studies with fluorescence microscopy will require the production of cysteine containing mutants of hgIIA with fluorescence labels attached such as Texas red. The attachment of these labels will need to be on the back of the protein on the opposite face from the interfacial binding surface and away from heparin binding sites. These fluorescence labels will permit the location of the internalised protein to be determined. In conjunction with such labels, incubating cells with $^{3}H$ arachidonic acid will permit the monitoring of the release of this inflammatory precursor and the fluorescence phospholipid analogue N-((6-(2,4-ditrophenyl)amino)hexanoyl)-1-hexadecanoyl-2-(4,4-difluoro-5,7-dimethly-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-sn-glycero-3-phosphoethanolaminen (PED6), which fluoresces when hydrolysed, will enable the localisation of the region of hydrolytic activity. In addition, the use of the non-hydrolysable membrane probe 1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine (DiIC12) will permit the monitoring of vesicle trafficking within the cell. Membrane budding has been noted in conjunction with movement of the hgV from the cytoplasm to the perinuclear membrane but was not seen with the IIA enzyme (20). Initially these investigations will be undertaken with HEK293 cells where the V3,31W hgIIA mutant will be expected to behave in a similar way to the hgV. Further to this it is envisaged increasing the cell lines used to incorporate a number of different types as hgV has been demonstrated to act in different locations in different cell types (11;20).

The results presented here demonstrate that the catalytic enhancement of hgIIA on neutral interfaces by tryptophan insertion is directly correlated to the binding affinities of the mutants. The fact that such a simple change, in evolutionary terms, to the proteins surface can lead to dramatically different binding properties and consequently to the proposed physiological activity of

the enzyme strongly argues that the hgIIA has evolved without an interfacial tryptophan residue to ensure its selectivity as an acute phase antibacterial protein.

The fluorescence properties of tryptophan are acutely sensitive to its local chemical environment including the presence of quenching agents. These properties of the tryptophan mutants were exploited in order use them as reporter groups to elucidate how the hgIIA protein binds to the membrane surface. The enzyme has been demonstrated here to bind to anionic PG SUVs with the hydrophobic collar of the interfacial binding surface tilted in relation to the plane of the membrane. Amino terminal helix 1 and helix 2 make the deepest penetration into the interfacial region of the bilayer and the active site slot is orientated perpendicular to the membrane surface (Chapter 4, figure 4.14A). This has been very recently confirmed by quantitative studies on mixed micelles using spin-labelling techniques and these observations are in marked contrast to the recently modelled group IB enzyme where the active site slot was seen to sit parallel to the plane of the membrane (131).

Significant protection and binding to the interface was seen for residues relatively distal to the active site entrance. These results coupled with the observations in Chapter 3, that the hgIIA enzyme seems to bind to anionic interfaces in a catalytically restricted manner, suggest that the membrane surface or the conformation of protein itself is affected by the very high affinity of this cationic enzyme for the such anionic surfaces. When, in Chapter 3, the charge on the enzyme was reduced by charge reversal mutagenesis (especially of the amino-terminal helix) the activity of the enzyme was observed to increase significantly against both SUVs and permeabilised Gram-positive bacteria. This suggests that the enzyme pays a "catalytic price" on anionic interfaces due to the requirement of such a high charge for the enzyme to be able to cross the peptidoglycan cell wall of Gram-positive bacteria and bind with high affinity to the cell membrane.

Importantly, the binding of hgIIA to the membrane surface was seen to alter according to the charge on the bilayer surface. As the negative charge on the

surface was reduced using 20mole%PG/PC SUVs the protein was seen to alter its orientation relative to the membrane surface and the hydrophobic collar sat flatter in relation to the plane of the membrane (Chapter 4, figure 4.14B). The change in orientation was observed as a rotation of the enzyme about a line between L20W and F70W and is hypothesised to be a reflection of the reduced electrostatic interactions present under these conditions. It is noteworthy that it cannot be ruled out that under these conditions the inserted tryptophan residues are effecting the enzyme's orientation, although it has been demonstrated here that the majority of the inserted residues have no effect on binding to a variety of PC containing interfaces.

Further work is underway at present to utilise cysteine mutants of the hydrophobic collar to which fluorescence probes can be attached. The fluorescence probe 2-(5-dimethylaminonaphth-1-yl-sufonamido)ethyl methanethiosulphonate (Dansyl-MTS) is the initial reagent chosen for these studies as it can be rapidly and specifically attached to thiol groups. This probe will then be used as a molecular dipstick to determine the depth of penetration of the protein into the bilayer, as has been done here with the tryptophan mutants. In addition to this qualitative study, it is envisaged obtaining a quantitative analysis of membrane binding using fluorescence resonance energy transfer with the dansyl labelled protein as the donor and a suitable fluorescein-labelled phospholipid as acceptor.

# Chapter 6 – References

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