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LOW-BARRIER HYDROGEN BONDING IN BOVINE PANCREATIC PHOSPHOLIPASE A₂

AND

SOMATIC INK4A-ARF LOCUS MUTATIONS: A SIGNIFICANT MECHANISM OF GENE INACTIVATION IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

The Degree Doctor of Philosophy in

The Graduate School of The Ohio State University

By

Ming Poi, B.S.

*****

The Ohio State University

2002

Dissertation Committee: Approved by

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Ohio State Biochemistry Program
ABSTRACT

This dissertation comprised of two independent parts. Part I is a study on low-barrier hydrogen bonds in bovine pancreatic phospholipase A\(_2\). Part II focuses on the somatic mutation screening of the \textit{INK4A-ARF} locus in head and neck squamous cell carcinomas.

Secreted phospholipases A\(_2\) (hereafter designated sPLA\(_2\)) are small (13 – 14 kDa) calcium-dependent lipolytic enzymes that belong to an extensive family of enzymes that facilitates the degradation of phospholipids at the \textit{sn}-2 ester bond. The active site of sPLA\(_2\) is constituted by an aspartate-histidine dyad, which coupled with the catalytic water molecule form a catalytic triad analogous to the well-studied serine proteases and lipases, except that the orientation of the imidazole ring in sPLA\(_2\) is reversed. In serine proteases, the catalytic triad consists of Asp, His, and Ser residues; and a proposed low barrier hydrogen bond (LBHB) is formed between Asp and His in both free and complexed states. Since sPLA\(_2\) also employs the general base mechanism, it would be interesting to know whether such a “LBHB” exists and plays an equivalent role. In this work NMR spectroscopy has been employed as a probe for the detection and characterization of a possible LBHB PLA\(_2\). The widely accepted features that are commonly associated with LBHB: (i) unusual downfield proton chemical shift, (ii)
fractionation number < 1.0, (iii) exchange rate protection factor > 10, and (iv) hydrogen bond strength ≤ (-7) kcal/mol.

Our results demonstrate that bovine pancreatic PLA2 exhibits three out four of the above criteria. An unusual downfield peak was observed at 17.8 ± 0.1 ppm in wild-type PLA2-inhibitor complex, which signified the presence of a deshielded proton. Wild-type sPLA2-inhibitor complex yielded a fractionation factor of 0.56 ± 0.06, and exchange rate protection factors of >100 at T = 8.5°C, and >40 at T = 25.0°C. However, the “putative LBHB” shows hydrogen bond strength of 4.6 kcal/mol, much less than the proposed -10 to -20 kcal/mol in energy. Furthermore, Histidine 15N-specific labeled experiments confirm that the strong H-bond is formed on the N^61 side of the catalytic histidine-48, and presumably with the transition state inhibitor, instead of between the catalytic aspartate-99 and histidine-48 as in the case of many serine proteases.

The p16INK4a (p16) tumor suppressor gene product is a negative regulator of cyclin-dependent kinase 4 (CDK4), which in turn positively regulates progression of mammalian cells through the cell cycle. In the present study, the mutational status of the p16 gene in 100 squamous cell carcinomas of the head and neck (SCCHN) was evaluated utilizing polymerase chain reaction (PCR) technology and a highly sensitive, nonradioactive modification of single-stranded conformational polymorphism analysis, termed “Cold” SSCP. Exons 1 and 2 of p16 were amplified utilizing intron-based primers or a combination of intron- and exon-based primers. A total of 27 SCCHN (27%) exhibited sequence alterations in p16. Somatic sequence alterations were identified in 22 of 100 (22%) tumor samples compared to the corresponding, patient-
matched, normal tissue. An additional five samples (5%) exhibited a G to A transition at
the first base of codon 148 in both tumor and patient-matched normal tissue, confirming
previous reports of this population polymorphism. Specific somatic alterations included
micro-deletions/insertions [9/22 (41%)], a micro-rearrangement [1/22 (5%)], and single
nucleotide substitutions [12/22 (56%)]. Secondly, several mutant p16 proteins that were
identified in the SCCHN were evaluated for biological significance. Of the eight
representative mutant proteins evaluated for CDK4 inhibitory activity, seven exhibited
reduced activity compared to wild-type P16, ranging from a 2-4-fold decrease in four
samples and a 25-40-fold decrease in three other samples. Overall, it appears that
somatic mutation of the p16 tumor suppressor gene is a significant event in the
development of SCCHN, which ultimately yields functionally deficient p16 proteins.
DEDICATION

To my Lord, Jesus Christ for all the blessings.

To my parents, husband Junan and son Erwin.
ACKNOWLEDGMENTS

None of the work described here would have been possible without the support from many people. I would like to acknowledge their efforts and give my sincere thanks to each and every one of them.

First of all, I would like to thank my parents, Tee Poi and Siew-Yoong Kok for their ceaseless love and support. I would not have accomplished what I have today if they had not supported my undergraduate studies in the United States. They not only provided me the opportunity to pursue my dream, but most importantly widen my horizon by offering their guidance and admirable perspectives of life.

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I am grateful to Dr. Chris Weghorst and Huijuan Song of the James Comprehensive Cancer Center for allowing me to utilize their laboratory and teaching me PCR and cold SSCP techniques during our collaboration. Their friendship are also very much appreciated.
I thank Dr. Chunhua Yuan for helping me in NMR spectroscopy, as well as offering his valuable advice on the PLA₂ project.

I would also like to thank Dr. Micheal Gelb of Washington University for providing me with the transition-state analogue (HK32) of PLA₂.

Finally, I like to thank everyone in Dr. Tsai's group for their friendship and help. Just to name a few, Ted Jiang, our lab manager; Dr. Hongxin Zhu, Dr. Xiaohong Liu, and Dr. Jinwoo Ahn, former students in this group; Dr. In-ja Byeon, and Li Zhao.
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PUBLICATIONS

1. *Somatic INK4a-ARF locus mutations: a significant mechanism of gene inactivation in squamous cell carcinomas of the head and neck.* Poi MJ; Yen T; Li J; Song H; Lang JC; Schuller DE; Pearl DK; Casto BC; Tsai MD; Weghorst CM. *Molecular Carcinogenesis* vol. 30, no. 1 (2001 Jan): 26-36.

2. *Structural basis of the anionic interface preference and kcat* activation of pancreatic phospholipase A2.* Yu BZ; Poi MJ; Ramagopal UA; Jain R; Ramakumar S; Berg OG; Tsai MD; Sekar K; Jain MK. *Biochemistry* vol. 39, no. 40 (2000 Oct 10): 12312-23.


FIELD OF STUDY

Major field: Biochemistry
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<tr>
<td>ARF</td>
<td>Alternative reading frame</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>CDK4</td>
<td>cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast performance liquid chromatography</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HN</td>
<td>head and neck</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear multiple quantum correlation</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum correlation</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-beta-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LBHB</td>
<td>low-barrier hydrogen bond</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear overhauser enhancement</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear overhauser and exchange spectroscopy</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SSCP</td>
<td>single-stranded conformation polymorphism</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)aminomethane</td>
</tr>
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Part I

THE STUDIES OF LOW-BARRIER HYDROGEN BONDING IN SECRETED BOVINE PANCREATIC PHOSPHOLIPASE A2
CHAPTER 1

INTRODUCTION

1.1 Introduction to phospholipase A$_2$ (PLA$_2$)

Historically, PLA$_2$ was first discovered in pancreatic juice and snake venom as early as the beginning of the 20th century. Since then, various intracellular and extracellular PLA$_2$s have been identified in many organisms. PLA$_2$ is a growing superfamily of lipolytic enzymes that catalyze the hydrolysis of phosphoglycerides at the $sn$-2 position, yielding free fatty acids and lysophospholipids (Figure 1.1).

![Figure 1.1 Reaction catalyzed by PLA$_2$s. R$_1$ and R$_2$ are hydrocarbon chains, which can vary in length and degree of saturation. PLA$_2$s specifically recognize the L-isomer at the $sn$-2 position. R$_3$ represents the head group. Some of the naturally occurring head groups in phosphoglycerides are choline, ethanolamine, inositol, serine, etc.](image)

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There are four major families of PLA\(_2\), which in turn can be divided into subfamilies in each case, and they are (i) secreted PLA\(_2\)s (sPLA\(_2\)s), (ii) cytosolic PLA\(_2\)s (cPLA\(_2\)s), (iii) Ca\(^{2+}\)-independent PLA\(_2\)s (iPLA\(_2\)s), and (iv) PAF acetylhydrolases (PAF-AH). Activity of specific PLA\(_2\) releases arachidonic acid (AA), which serves as the precursor for the biosyntheses of eicosanoids, such as prostagladins (PGs), leukotrienes, etc.; as well as lysophospholipid-derived mediators, such as platelet-activating factor (PAF), and lysophosphatic acid. These molecules have been found involved in many biochemical reactions, including sequestering inflammatory responses, lipid digestion, cell proliferation, exocytosis, antibacterial defense, anti-coagulation, etc. Therefore, overproduction or deficiency of PLA\(_2\)s can cause a variety of diseases and tissue disorders, such as inflammatory diseases (Kudo, et al., 1993). Ischemia (Lauritzen, et al., 1994; Koike, et al., 1995), and atherosclerosis (Ivandic, et al., 1999; Tietge, et al., 2000).

PLA\(_2\)s can exist intracellularly and extracellularly. Low concentrations of intracellular PLA\(_2\)s have been detected in almost all mammalian cells. The purification and cloning of numerous PLA\(_2\)s have clearly demonstrated the differences between sPLA\(_2\)s and cPLA\(_2\)s. The sPLA\(_2\)s share high sequence homology (~70%), and have relatively low molecular weight (14-16 kDa). sPLA\(_2\)s are Ca\(^{2+}\)-dependent with a millimolar range requirement for the cofactor. A more detailed characterization of the sPLA\(_2\)s is addressed later in this chapter. In contrast, cPLA\(_2\)s have higher molecular weights (40-110 kDa), and are either Ca\(^{2+}\)-independent or only require the cofactor in the micromolar range for activity. In addition, cPLA\(_2\)s preferentially act on sn-2-arachidonoyl phospholipids in vitro, whereas sPLA\(_2\)s do not display such selectivity.
Leslie et al., 1988; Clark et al., 1990; Clark et al., 1991; Hazen et al., 1990; Ackermann et al., 1994).

Structural and functional studies of the cPLA₂s are more difficult than sPLA₁s due to their relatively larger size and limited availability. Therefore, in depth structural and functional investigation of PLA₂s have been conducted primarily on sPLA₁s, particularly the group I and II, which include the pancreatic and venom sPLA₁s. All data obtained for the low-barrier hydrogen bonding studies in this dissertation were performed on bovine pancreatic sPLA₁ (Figure 1.2).

1.2 Secretory PLA₁s

sPLA₁s are Ca²⁺-dependent interfacial enzymes that have a low molecular weight of approximately 14 to 16 kDa. To date, at least ten structurally related isozymes have been found in mammals (Figure 1.3; IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII). Many sPLA₁ structures (zymogen, free enzymes, and protein/ligand complexes) have been obtained using X-ray crystallography and nuclear magnetic resonance (NMR), and there are over forty sPLA₁ entries in the Protein Data Bank (Dijkstra, et al., 1981; Dijkstra et al., 1983; Scott, et al., 1990; Westerlund et al., 1992; Sekar, et al., 1997b; Yuan, et al., 1999; Steiner et al., 2001). However, the pancreatic sPLA₁s, group IB sPLA₁ are among the first studied and well-characterized phospholipases. Extensive studies have been conducted in our laboratory, as well as other research groups on the enzymatic mechanism and structure-function relationship of pancreatic sPLA₁s. The solution structure of the free bovine pancreatic sPLA₁ is shown in figure 1.4 (Yuan, et
Figure 1.2 The sequence of the recombinant gene for bovine pancreatic sPLA₂
(Adapted from Noel & Tsai, 1989).
Figure 1.2
Figure 1.3  Schematic structures of mammalian secretory phospholipase A\textsubscript{2}s (sPLA\textsubscript{2}s).
Figure 1.3

<table>
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<tr>
<th>Isozyme</th>
<th>Structure</th>
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<tr>
<td>sPLA₂-IB</td>
<td>prosequence Ca²⁺-binding site catalytic histidine pancreatic loop</td>
<td>14</td>
</tr>
<tr>
<td>sPLA₂-IIA</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>sPLA₂-IIIC</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>sPLA₂-IIID</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>sPLA₂-IIIE</td>
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<td>14</td>
</tr>
<tr>
<td>sPLA₂-IIIF</td>
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<td>15</td>
</tr>
<tr>
<td>sPLA₂-V</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>sPLA₂-X</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>sPLA₂-III</td>
<td></td>
<td>10</td>
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</tbody>
</table>

No. of Cys:

- sPLA₂-IB: 14
- sPLA₂-IIA: 14
- sPLA₂-IIIC: 16
- sPLA₂-IIID: 14
- sPLA₂-IIIE: 14
- sPLA₂-IIIF: 15
- sPLA₂-V: 12
- sPLA₂-X: 16
- sPLA₂-III: 10
Figure 1.4  Ribbon representation of bovine pancreatic sPLA₂ solution structure showing the secondary structures, seven disulfide bonds, and the His₄₈-Asp₉₉ catalytic dyad. Reproduced from Yuan et al., 1999.
al., 1999). Some of the common structural features among the sPLA\textsubscript{2}s are: (i) an \(\alpha\)-helical N-terminus, which often contains lipophilic residues such as tryptophan and valine; (ii) a \(\text{Ca}^{2+}\)-binding loop with a typical glycine-rich stretch; (iii) a conserved histidine residue in the active site, along with aspartic acid and tyrosine residues; and (iv) 12 to 16 cysteine residues, forming a number of disulfide bonds.

Pancreatic sPLA\textsubscript{2}s belong to the group I isozymes. These enzymes are synthesized and stored as an inactive form (zymogen) in the secretory granules in the pancreas. After being secreted into the gastrointestinal tract, the N-terminal prosequence is cleaved by trypsin, yielding a mature/fully active enzyme. The mature enzyme strongly prefers aggregated substrates, and performs inefficiently on monomeric substrates. This phenomenon is referred to as interfacial activation (Figure 1.5).

1.2.1 Catalytic mechanism of pancreatic sPLA\textsubscript{2}

Currently, there are two proposed catalytic mechanisms of pancreatic sPLA\textsubscript{2}. The first mechanism was proposed by Verheij et al. in 1980. The Asp-His dyad of pancreatic sPLA\textsubscript{2} along with a catalytic water molecule form a “triad”, a mechanism analogous to the mechanism for serine proteases (Asp-His-Ser triad). Here, H48 acts as a general base. The rate-limiting step in this mechanism is presumed to be the formation of the tetrahedral intermediate upon the protonation of \(N^\delta1\) of H48.

An alternative mechanism was brought forward later by Yu et al. in 1998. In this mechanism a \(\text{Ca}^{2+}\)-coordinated oxyanion is formed upon the attack of the catalytic water molecule, which is connected to \(N^\delta1\) of H48 through another water molecule (two-water molecule mechanism). During the formation of the tetrahedral intermediate, the
imidazole ring of H48 is also protonated. Another key difference between the two mechanisms is that the rate-limiting step in the Ca$^{2+}$-coordinated mechanism is the decomposition of the tetrahedral intermediate (Figure 1.6).

1.2.2 Structural characteristics of bovine pancreatic sPLA$_2$

As shown in figure 1.4, $\alpha$-helices and antiparallel $\beta$-strands constitute about 50% and 10% of the protein, respectively. The protein is "compact" and rigid as it is held together by seven pairs of disulfide bonds. The catalytic Asp99-His48 pair coordinated to a water molecule, forming a "triad" in the catalytic core. A calcium ion (an obligatory cofactor for catalysis) binds to the calcium-binding loop (Y25-T36), and it is coordinated to five oxygen ligands contributed by the protein. Two oxygen ligands come from the Asp49 side chain, and the rest from the backbone carbonyl of residues 28, 30, and 32. A "hydrophobic channel" constituted by residues Leu2, Phe5, Ile9, Ala102, and Phe103 connects the catalytic core to the $i$-face of the enzyme, the region where interfacial binding of the enzyme to its substrates takes place.

Another important feature of the protein is the extensive hydrogen-bonding network, which has been shown to be crucial in maintaining the structural integrity of the enzyme (Yuan et al., 1999). Some of the key participants of the network are the N-terminus (A1 and Q4), H48, D99, Y52, Y73, and a number of water molecules. Figure 1.7 shows the hydrogen-bonding network in the solution and crystal structures of bovine pancreatic sPLA$_2$. 

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Figure 1.5  Interfacial activation behavior observed in porcine pancreatic PLA\(_2\). The activity of mature sPLA\(_2\)(a), and its zymogen(b) as a function of diheptanoyllecithin concentration. CMC is the abbreviation for critical micelle concentration. Reproduced from Pieterson et al.. 1974.
Figure 1.5

Enzyme Activity (Units/mg)

CMC  L-DHpL (mM)

(a)

(b)

1 2 3 4

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Figure 1.6  Proposed catalytic mechanisms of pancreatic sPLA₂.  A. Catalytic “triad” mechanism analogous to the catalytic mechanism of serine proteases, and H48 acts as a general base.  B. Ca²⁺-coordinated mechanism (two-water molecule mechanism). w5 is the catalytic water molecule, and w6 links the catalytic water molecule to the imidazole ring of H48 (reviewed in Berg et al., 2001).
1.2.3 The Hydrogen-bonding network in the catalytic core

The hydrogen-bonding network in the catalytic core (Figure 1.7) links the active sites to the interfacial binding site of the protein. It was initially suggested that these hydrogen bonds are important for interfacial catalysis (Verheij et al., 1981). However, extensive site-directed mutagenesis studies, together with scooting mode kinetics, and crystal structures of mutant enzymes of the highly conserved residues in the network revealed that these hydrogen bonds are not crucial for interfacial activation, and in fact they only hold a modest role in catalysis. It has been demonstrated that the hydrogen-bonding network is important in maintaining the structural integrity of the enzyme (Dupureur et al., 1992; Sekharudu et al., 1992; Kumar et al., 1994; Sekar et al., 1997a). In addition, the hydrogen-bonding network is more clearly defined in complex structures than in free structures. Therefore, it is suggested that the hydrogen-bonding network is probably the scaffold that provides stability to the catalytic Asp-His dyad during catalysis.

1.3 Introduction to hydrogen bonds.

Hydrogen bonding is a ubiquitous feature of chemical constitution and reactivity. It is the key element that contributes to the structure and properties of some of the most important building blocks of life, for instance water, the DNA helical structures, and protein secondary structures. In general terms, a hydrogen bond $A—H \ldots B$ is often described as an electrostatic attraction between the positive end of the bond dipole of $AH$ (hydron donor) and a center of negative charge on $B$ (hydron acceptor). The atom/group
Figure 1.7 The hydrogen-bonding network in sPLA$_2$. (Top) Structural details in the solution structure of bovine pancreatic sPLA$_2$ (Yuan et al., 1999). (Bottom) Structural details derived from crystal structures (Dijkstra et al., 1981; Verheij et al., 1981; Scott & Sigler, 1994)
Figure 1.7
A should be sufficiently electronegative so that the A—H bond is relatively polar, and B usually owns a lone pair of electrons. The hydrogen bond donor AH, and the hydrogen bond acceptor B are commonly nitrogen, oxygen, and fluorine with large dipole moments. Carbon can participate in hydrogen bonding under certain circumstances, for instance in the presence of electron-withdrawing group and in aromatic \( \pi \)-system. Stronger hydrogen bonds are generally found with cations or anions, for instance between acids and their conjugate bases (\( A-H \ldots A^- \)), or bases and their conjugate acids (\( B^+ - H \ldots B \)). It is generally assumed that a regular hydrogen bond in biological system involves 1 to 4 kcal/mol of energy (Fersht et al., 1985; von Hippel & Berg, 1989; Jeffrey & Saenger, 1991). Hydrogen bonds formation cause evident physicochemical changes to the hydrogen donor and acceptor – molecular dimensions, energetics, vibrational frequencies, and electron distribution (Table 1.1).

Hydrogen bond lengths are commonly described in terms of the distance between the two heavy atoms involved in the hydrogen-bonding, \( R_{A...B} \) because it is often difficult to locate the hydrogen atom, and \( R_{A...B} \) spans the range of 2.25 to 3.10 Å (Hibbert & Emsley, 1990). Neutron diffraction has been a powerful technique to pinpoint the location of hydrogen atom in smaller molecules, but not in complex entities like proteins. The criterion for the presence of hydrogen bonds is based on the van der Waals radii. If \( R_{A...B} \) is less than the sum of the van der Waals radii of the two heavy atoms involved, \( \Sigma_w(A+B) \), then it seems reasonable to assume that a hydrogen bond is present.
| Dimensions            | Bond length, $R_{A...B}$  | Proton location, $R_{A-H}$ | Bond angle, $\angle_{AHB}$ | Energetics               | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
|----------------------|---------------------------|-----------------------------|-----------------------------|---------------------------|------------------------|------------------------|------------------------|--------------------------|----------------------|--------------------------|----------------|------------------------|----------------|--------------------------|----------------|------------------------|----------------|------------------------|----------------|------------------------|----------------|------------------------|----------------|------------------------|----------------|------------------------|
|                      |                           |                             |                             | Energetics               | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
| X-ray crystallography|                           |                             |                             | Neutron diffraction      | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
| Calorimetry          |                           |                             |                             | IR spectroscopy, $\Delta V_{AH}$ | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
| IR spectroscopy      |                           |                             |                             | Ion cyclotron resonance spectrometry | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
| Theoretical calculations | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
| IR spectroscopy      | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
| Raman spectroscopy   | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |
| NMR spectroscopy     | Bond energy, $E_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AH} / \Delta V_{AD} \) | Vibrational properties | Fractionation factor, $\phi$ | Potential energy well | $\delta_{AHB}$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | Potential energy well | $\delta_{AHB}$ | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ | \( \Delta V_{AH} \)  | \( \Delta V_{AD} \) | \( \delta_{AHB} \) | Fractionation factor, $\phi$ |

**Table 1.1** The parameters of hydrogen bonds, A — H ... B
1.4 **Potential energy wells**

Hydrogen bonds are generally divided into 3 types based on the relative position of H or D between the two heavy atoms (Figure 1.8). For a weak hydrogen bond the potential energy barrier between the minima will be high, and therefore the proton is confined to the well of the parent heavy atom to which it is covalently bonded. The zero-point energy of a deuteron that is engaged in weak hydrogen bonding is lower because deuteron is double in mass. However, in the case of weak hydrogen bonding, it is generally assumed that the difference in isotope has no effect on the bond length. For strong or low-barrier hydrogen bonds, the zero-point energy of H may be above or close to the top of the barrier, while of D is confined within the well. As the hydrogen bond strength increases, the covalent A—H moiety lengthens, while the noncovalent moiety H ... B shortens. The proton is delocalized, and moves freely between the two heavy atoms. In the case of very strong or single-well hydrogen bonds, both H and D have zero-point energy above the barrier. As the two heavy atoms continue to approach each other, the energy barrier between the wells gradually diminishes. For single-well hydrogen bonds, the average location of the proton is the midpoint position between the two heavy atoms.

1.5 **Hydrogen bonding and enzyme catalysis**

Hydrogen bonds are one of the major forces that maintain the three-dimensional structure of enzymes at all states – free enzyme, transition state(s), intermediate(s), and substrate-bound/complex. Hydrogen bonds are partly accountable for the high catalytic
Figure 1.8  The three types of A—H ... B hydrogen bond.
<table>
<thead>
<tr>
<th>Type</th>
<th>Potential energy well</th>
<th>Energy (kcal mol(^{-1}))^a</th>
<th>Energy (kJmol(^{-1}))^b</th>
<th>R(_{A...B}) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak</td>
<td>B ...... A</td>
<td>1 - 3</td>
<td>10 - 50</td>
<td>2.7 - 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>B ...... A</td>
<td>7 - 25</td>
<td>50 - 100</td>
<td>2.5 - 2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very strong</td>
<td>B ...... A</td>
<td>&gt; 25</td>
<td>&gt; 100</td>
<td>2.3 - 2.4</td>
</tr>
</tbody>
</table>

\(^{a}\) Mildvan et al., 1999  
\(^{b}\) Hibbert & Emsley, 1990

Figure 1.8
efficiency and the specificity of enzymes. Low-barrier hydrogen bonds have been proposed (Gerlt & Gassman, 1993; Cleland & Frey, 1994), and later supported by studies done on many enzymes, including proteases (Frey et al., 1994; Cassidy et al., 1997), isomerases (Harris et al., 1997; Zhao et al., 1997), etc. to be responsible for the low activation energy in some enzyme catalysis. The proposal has been embraced by many because it provides a reasonable explanation to how an enzyme can overcome the large $\Delta G^\circ$ (energy difference between the intermediate and the enzyme-substrate complex) for the abstraction of $\alpha$-hydrogen to a carbonyl group. While evidence in support of this postulation is accumulating, the unusual role of LBHBs in enzymatic function and even its existence have also been under intensive debate (Guthrie et al., 1996; Warshel et al., 1995; Warshel & Papazyan, 1996; Ash et al., 1997; Garcia-Viloca et al., 1997).

The purpose of this project is to employ NMR spectroscopy as a probe for the detection and characterization of a possible LBHB in bovine pancreatic sPLA$_2$. 

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CHAPTER 2

MATERIALS AND METHODS

Construction of pET25b-(m)-prosPLA₂ expression vector. Expression vector pET25b purchased from Novagen (Madison, WI) was modified at its multi-linker site in order to create a 5’ EcoRI site and 3’ BamHI site. The modified plasmid is named pET25b-(m). XL I blue Escherichia coli (E. coli) strain was used in all recombinant DNA experiments.

Site-specific mutagenesis. Quickchange™ method (Stratagene) was employed in all site-directed mutagenesis experiments using pET25b-(m)-prosPLA₂(wild-type) as template. Oligonucleotides were obtained from IDT inc. (Coralville, IA). The sequences of the oligonucleotides (in complementary sets) were as following:

5’-CAT TTA TTT GTA ACT GCA ATC GTA ACG CTG C-3’ for D99N;
5’-CCT TAT AAC AAA GAA GCC AAG AAT CTT GAT AAA-3’ for H115A.

Expression and purification of bovine pancreatic sPLA₂. The recombinant sPLA₂ and mutants proteins were isolated from the E. coli expression host, BL21(DE3)[plysS].

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<table>
<thead>
<tr>
<th>Solution</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>100 mM Tris, 5 mM EDTA, 0.5% Triton X-100, 50 mM NaCl, pH 7.7</td>
</tr>
<tr>
<td>Urea/Sulfite solution</td>
<td>8 M urea, 0.3 M Na₂SO₃, pH 8.0</td>
</tr>
<tr>
<td>NTSB (2-nitro-5-(sulfonthio) benzoate)</td>
<td>1 M Na₂SO₃, 50 mM Ellman's reagent, stirred (under aeration) until yellow color, pH 8.0</td>
</tr>
<tr>
<td>Solubilizing refolding buffer</td>
<td>8 M urea, 50 mM borate, 5 mM EDTA, 4 mM reduced glutathione, 2 mM oxidized glutathione, pH 8.4</td>
</tr>
<tr>
<td>Diluting refolding buffer</td>
<td>50 mM borate, 5 mM EDTA, 4 mM reduced glutathione, 2 mM oxidized glutathione, pH 8.4</td>
</tr>
<tr>
<td>SSFF column buffer</td>
<td>10 mM succinate, pH 4.5-4.6</td>
</tr>
<tr>
<td>QSFF column buffer</td>
<td>10 mM Tris, pH 8.4-8.5, filtered and degassed.</td>
</tr>
</tbody>
</table>

Table 2.1. Summary of solutions and buffers for sPLA₂ purification
carrying the pET25b-(m)-prosPLA2 plasmid. Since the plasmid contained a leader sequence, sPLA2 was expressed in the host cells as inclusion bodies. Routine preparation of WT or mutant sPLA2 was carried out at 10-liter scale of rich media (19 g yeast extract, 12 g tryptone/ liter; pH 7.5). Two 100 ml LB medium in 500 ml Erlenmeyer flasks containing 0.2 mg/ml ampicillin and 0.015 mg/ml chloramphenicol were inoculated with fresh transformants of BL21(DE3)[plysS] carrying pET25b-(m)-prosPLA2 plasmid, and shaken at 200 -250 rpm, 37 °C overnight (10-12 hrs). Each liter of rich medium contained 20 ml of overnight culture, 0.2 mg/ml ampicillin, 0.015 mg/ml chloramphenicol, and 10 ml of 1 M MgSO4. The inoculated media were shaken at 37 °C until A600 reached 0.6 -0.8. Sterile filtered IPTG (final concentration of 1 mM) was then added to induce the expression of sPLA2 protein. An additional 1 ml of 0.2 mg/ml ampicillin was also added to each 1 liter of medium. The cells were grown for additional 8 hrs, and harvested by centrifuging at 9,000 rpm at 4 °C for 15 min. The average cell yield was 3-4 g/l of wet cell pellet. Cell pellet was stored at (-20) °C.

Solutions and buffers used in the process of purification were listed in Table 2.1 The frozen cell pellet was stirred vigorously at RT in 250 ml of lysis buffer containing 30 mg of lysozyme. As the pellet was broken up, PMSF (phenyl methyl sulfonyl fluoride) of a final concentration of 0.2 mM was dissolved in ethanol, and added to inhibit protease activity. After stirring for ½ - 1 hr, the partially homogenized suspension was chilled on ice, and sonicated at 70% output and 80% power, for 45 s/cycle with a 5-minute interval until homogeneity. The suspension was then centrifuged at 9,000 rpm. 4 °C for 15 min. The pellet was resuspended in 150 ml lysis buffer containing 30 mg lysozyme, and stirred vigorously at RT for an additional ½ hr and another 5-10 cycles of sonication were
applied to achieve complete lysis. The suspension was centrifuged at 9,000 rpm, 4 °C for 15 min. The pellet was solubilized thoroughly in 130 ml of urea/sulfite solution by stirring vigorously for 2-3 hrs at RT. 20 ml of NTSB solution was added and stirred for additional 30 min at RT. The mixture was transferred into dialysis tubing with a molecular weight cutoff of 6,000 to 8,000 daltons, and dialyzed against 8 liters of distilled water at 4 °C overnight. The distilled water was changed at least once. The mixture was then dialyzed against 8 liters of distilled water containing 20 ml of glacial acetic acid for additional 4 hrs. The content of the dialysis tubing was centrifuged at 9,000 rpm, 4 °C for 15 min.

The pellet from the preceding step was solubilized completely by stirring in 75 ml of freshly prepared solubilizing refolding buffer (for 2-3 hrs). Upon complete solubilization, the mixture was diluted with 225 ml of freshly prepared (chilled) diluting refolding buffer by dripping from a pump over a 2-3 hr period. The mixture was stirred for an additional ½ hr before left in the cold room overnight. The pH of the mixture was slowly adjusted to 5.0-5.2 with dilute glacial acetic acid (about 1 M). The precipitate resulting from acidification was removed by centrifugation at 9,000 rpm, 4 °C for 15 min. The supernatant was transferred into dialysis tubing with a molecular weight cutoff of 6,000-8,000 daltons, and dialyzed against 8 liters of distilled water at 4 °C overnight with at least one change of dialysate.

The 10-ml SSFF (S Sepharose Fast Flow, strong cation exchanger) column was equilibrated with 10 mM succinate buffer at pH 4.5-4.6 centrifuged at 9,000 rpm, 4 °C for 15 min to remove any residual precipitate. The pH of the dialyzed supernatant was adjusted to the pH of the pre-equilibrated SSFF column, centrifuged at 9,000 rpm, 4 °C
for 15 min to remove any residual precipitate. The supernatant was then loaded onto the SSFF column. The column was washed with at least 100 ml SSFF buffer, and proPLA₂ protein was eluted with a manual gradient of NaCl from 0 to 400 mM (150 ml of each low and high salt buffer) at approximately 2 ml/min. ProsPLA₂ usually came out at 160-170 mM NaCl. Fractions from the peak were combined, and dialyzed against 8 liters of distilled water at 4 °C overnight. The pro-enzyme was lyophilized and stored in the (-80) °C freezer. A QSFF (Q Sepharose Fast Flow, strong anion exchanger) column was equilibrated with 10 mM Tris buffer, pH 8.4-8.5 a day before the activation of the pro-enzyme by trypsin cleavage. A 2mg/ml prosPLA₂ protein solution was prepared in QSFF buffer, and pH of the solution was adjusted to 8.0. 0.1% (w/w) trypsin was added to digest prosPLA₂. 1 mg/ml of sPLA₂ and trypsin solution have A₂₈₀ of 1.3 and 1.44, respectively. The digestion was terminated with PMSF in ethanol with final concentration of 0.2 mM. Typical digestion time was approximately 60 min at RT. Then, 2x – 3x volume of the QSFF buffer was added to the enzyme mixture to reduce ionic strength, and pH adjusted to the pH of the pre-equilibrated column before being loaded onto the column. After a washing step with at least 100 ml QSFF buffer, sPLA₂ was eluted from the column using a Pharmacia FPLC system. A gradient of 0-250 mM NaCl was applied, and sPLA₂ typically was eluted around 50-100 mM NaCl. The fractions were pooled and transferred into dialysis tubing with a molecular weight cutoff of 3,000 daltons, and dialyzed overnight against 8 liters of distilled water. The mature enzyme was then lyophilized and stored at (-20) or (-80) °C.
Isotopic labeling of sPLA₂. The procedures to express uniform $^{15}$N-labelled sPLA₂ were similar to the procedures outlined previously, except M9 minimal medium with a $^{15}$N source was used instead. Every liter of the $^{15}$N growth medium contained 970 ml of M9 medium (6.78 g of Na$_2$HPO$_4$, 3 g of KH$_2$PO$_4$, 1.0 g of $^{15}$NH$_4$Cl, 0.5 g of NaCl, pH 7.4, autoclaved), 10 ml of 40 % glucose (sterile filtered), 2 ml of 1 M MgSO$_4$ (autoclaved), 0.1 ml of 1 M CaCl$_2$ (autoclaved), 0.2 g of sterile filtered ampicillin, and 0.015 g of sterile filtered chloramphenicol. 20 ml of overnight culture (10 – 12 hrs) were used to inoculate each liter of medium. However, the overnight culture was first centrifuged, and resuspended in M9 medium before the inoculation in order to remove LB medium. The cell culture was grown to $A_{600} = 0.6 - 0.8$ at 37 °C, and induced by IPTG of 1 mM final concentration. The cells were then grown for additional 10 hrs.

The procedures to prepare selectively-labeled protein were essentially the same except synthetic rich (SR) medium was used instead of M9. 950 ml of double distilled water contained 0.50 g of L-alanine, 0.40 g of L-arginine, 0.40 g of L-aspartic acid, 0.05 g of L-cysteine, 0.40 g of L-glutamine, 0.65 g of L-glutamic acid, 0.55 g of glycine, 0.10 g of L-histidine, 0.23 g of L-isoleucine, 0.23 g of L-leucine, 0.42 g of L-lysine-HCl, 0.25 g of L-methionine, 0.13 g of L-phenylalanine, 0.10 g of L-proline, 2.10 g of L-serine, 0.23 g of L-threonine, 0.17 g of L-tyrosine, 0.23 g of L-valine, 1.50 g of NaAc, 1.50 g of succinic acid, 0.50 g of NH$_4$Cl, 0.85 g of NaOH, 10.50 g of K$_2$HPO$_4$, 0.50 g of adenine, 0.65 g of guanosine, 0.20 g of thymine, 0.50 g of uracil, and 0.20 g of cytosine.

For selective labelling of a specific amino acid, the corresponding non-labeled amino acid was replaced by its isotope, and the amount of the isotope added was reduced by 0 – 10 fold. For selective histidine labeling, 0.05 g of labeled histidine was added per liter of...
SR medium. The above medium was autoclaved, and the following ingredients were added to each 1 liter of medium: 50 ml of 40 % glucose, 4 ml of 1 M MgSO₄, 1 ml of 0.01 M FeCl₃, 1 ml of metal ions solution (2 mg of CaCl₂·H₂O, 2 mg of ZnSO₄·7H₂O, 2 mg of MnSO₄·H₂O), 10 ml of vitamins/nutrient solution (50 mg of tryptophan, 50 mg of thiamine, 50 mg of niacin, 1 mg of biotin), 0.2 g of ampicillin, and 0.015 g of chloramphenicol.

NMR samples and NMR experiments. NMR samples typically were 0.4 – 0.7 mM protein, except 0.15 mM for H115A, which aggregated at higher concentration. 5 to 10 % (v/v) of DMSO-d₆ was added to the samples, unless otherwise indicated. The reasons for adding DMSO-d₆ are: (i) for field frequency locking; (ii) enable the experiments to be conducted at low temperature (as low as 0 °C) without freezing the samples; and (iii) solvent for the transition state analogue. The phosphonate transition-state analogue HK32 was dissolved in deuterated DMSO to make a stock solution of = 25-50 mM, and the final [inhibitor]/[protein] ratio was typically made to 1-2. Exception for the deuterium exchange experiments for fractionation determination. NMR protein samples were prepared in 80 % H₂O / 10 % D₂O / 10 % DMSO, with 200 mM NaCl and 50 mM CaCl₂, pH 5.0 – 6.5.

Since a narrower line width was obtained at 288 K, which in turn improved the signal-to-noise ratio, all NMR experiments were carried out at 293K on a Bruker DMX-600 spectrometer unless stated otherwise. The jump-return method (Plateau and Gueron, 1982) was used to suppress the water signal with the carrier frequency set on the water signal and the delay between the pulses optimized to achieve maximum excitation at 18
ppm. When needed a continuous-wave with low power (25 dB) was applied to the f3
($^{15}$N) channel to achieve $^{15}$N decoupling.

The pH titrations were performed gradually over the pH range from 4 to 12. The
pH of the protein sample was lowered from pH 12 and checked by one-dimensional $^1$H
NMR to make sure that the protein was not denatured under highly basic conditions. The
pKa of H48 in the absence of HK32 was determined by pH titration monitored by $^1$H-$^{13}$C
HMQC on a $^{13}$Cε1- His specific-labeled wild type enzyme. The corresponding pKa in the
HK32-protein complex was determined by pH titration monitored by jump-return by
integrating the downfield signal (Cassidy, et al., 1997). The pKa value was obtained by
fitting to the following modified Henderson-Hasselbalch equations using the Marquardt-
Levenberg nonlinear least-squares algorithm (SigmaPlot, Jandel Scientific):

$$\delta_{\text{obs}} = \delta_A + (\delta_{\text{HA}} - \delta_A) \frac{10^{pK_a}}{10^{pK_a} + 10^{pH}}$$

(1)

$$I_{\text{obs}} = I_0 + \Delta I \frac{10^{pK_a}}{10^{pK_a} + 10^{pH}}$$

(2)

Here, $\delta_{\text{obs}}$ is the observed peak chemical shift; $\delta_A$ is the chemical shift of the unprotonated
imidazole; $\delta_{\text{HA}}$ is the fully protonated imidazole; $I_{\text{obs}}$ is relative peak intensity observed;
$I_0$ is the maximum relative peak intensity of the protonated imidazole, which is
normalized to 1; $\Delta I$ is the difference between the maximum and minimum relative peak
intensities, which is (-1).

For the measurement of the fractionation factor, $k_{\text{eq}}$, and pKa, duplicate
experiments were performed to ensure consistency of the data. Data were processed
using XWINNMR software (Bruker). Typically, zero-filled and window multiplication were applied prior to Fourier transformation. Proton chemical shifts were calibrated against the external standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

**NMR criteria for low-barrier hydrogen bonds.**

(A) **Proton chemical shift**

A.1 **Theory**

It has been established from the correlations of hydrogen bond lengths determined by X-ray diffraction with the chemical shifts of proton engaged in hydrogen bonding that as a hydrogen bond length decreases, the corresponding proton becomes more deshielded, and therefore causes a downfield shift in proton resonance. The deshielding effect is caused by the lengthening of the covalent bond A—H of the A—H ... B hydrogen bond. Lengthening of the bond weakens the shielding influence provided by the σ bond electrons on the donor heavy atom.

A.2 **Method**

NMR experiments for detection and measurement of the proton resonance were conducted at low temperatures to suppress signal broadening due to proton exchange with the solvent. The line width of the proton resonance decreases as the temperature is lowered, until it reaches a minimum, and broadens again due to dipolar effects. All NMR samples were prepared in H₂O (with 10 % D₂O, v/v), therefore, suppression of the intense water signal was crucial in order to detect the weak downfield resonance. The jump-return pulse scheme was found to be satisfactory, and was used in all ¹H one-dimensional spectra, whenever the detection of the downfield signal was desired.
(B) Fractionation factor

B.1 Theory

A solvent-exchangeable proton on an enzyme can be expressed as:

\[
\text{Enzyme-H} + D_{\text{solvent}} \rightleftharpoons \text{Enzyme-D} + H_{\text{solvent}}
\]

The fractionation factor \( \phi \), of the corresponding proton is defined as the equilibrium constant for the exchange of deuterium from the solvent onto this position:

\[
\phi = \frac{[\text{Enzyme-D}] [H_{\text{solvent}}]}{[\text{Enzyme-H}] [D_{\text{solvent}}]}
\]

Measurement of the equilibrium distribution of deuterium relative to hydrogen atoms in a proton exchangeable site is informative about the nature of the environment of the corresponding proton. When a proton is engaged in a weak (regular) hydrogen bond, \( \phi \) is approximately 1. As the hydrogen bond gets stronger, the value of \( \phi \) gets smaller (< 1.0). The rationale behind this observation is that when a proton is engaged in a hydrogen bond, the covalent bond between the proton and its parent heavy atom has a lower effective force constant. The covalent bond therefore vibrates in a wider and shallower potential energy well at a lower zero-point energy. As for a deuteron, it is \( \sqrt{2} \)-fold less affected by the decrease in the effective force constant because of its two-fold greater in mass; thus the zero-point vibrational energy of the hydrogen-bonded deuteron decreases less than a corresponding proton. As a consequence, the presence of a deuteron in the
hydrogen bond is less favorable as compared with a proton. Accordingly, the stronger the hydrogen bond, the more the deuteron is disfavored at this position, yielding a fractionation factor that is $< 1$. However, the above increasing relationship (bond distance vs. fractionation factor) will reach a minimum point before an inverse relationship takes place that is when a very strong single-well hydrogen bond is formed. Such very strong hydrogen bonds thus far have not been reported in proteins.

B.2 Method

Determination of fractionation factor for solvent exchangeable proton resonance was achieved by comparing peak intensity of the corresponding $^1$H NMR resonance at equilibrium in mixed H$_2$O-D$_2$O solution. Quantitative measurement of peak intensity was normalized to an upfield methyl resonance.

Initially, the protein solution obtained from the final step of the purification process was concentrated to approximately 5 mg/ml. One ml of protein solution was dispensed into each 2-ml Eppendorf tubes, and lyophilized to make an equal-mass batch of protein samples. These protein samples were re-dissolved into a series of H$_2$O-D$_2$O buffer mixtures to make a final volume of 600 µl in every sample. The protein samples of different H$_2$O-D$_2$O solutions were then incubated for at least 12 hours at room temperature for complete equilibration of the exchangeable proton of interest. Complete equilibration was checked by repeated experiments after several weeks. Fractionation factor was derived by fitting NMR data to the following equation:

$$\frac{1}{I} = \frac{\phi}{I_{\text{max}}} \left( \frac{1 - X}{X} \right) + \frac{1}{I_{\text{max}}}$$
In which $\phi$ is the fractionation factor, $I$ is the observed peak intensity at a given mole fraction of H$_2$O, $X$; $I_{\text{max}}$ is the maximal peak intensity when $X=1.0$, and $(1-X)$ is the mole fraction of D$_2$O.

(C) Proton exchange rate and protection factor

C.1 Theory

The protection factor (PF) is expressed in the following equation:

$$\text{PF} = \frac{k_{\text{intrinsc}}}{k_{\text{ex}}}$$

Where, $k_{\text{ex}}$ is the pseudo-first-order exchange rate constant for the hydrogen-bonded proton with solvent protons, and $k_{\text{intrinsc}}$ is the pseudo-first-order exchange rate constant for the same proton in the absence of hydrogen bonding. The proton exchange rate constant with solvent can be experimentally determined using NMR spectroscopy. This kinetic parameter of the proton is determined from the temperature dependence of the width of the corresponding proton resonance in $^1$H NMR spectra.

A proton engaged in hydrogen bonding has a slower exchange rate with the solvent protons. Therefore, PF is expected to be greater than unity for hydrogen-bonded proton. The value of the proton exchange rate constant is obtained by incorporating the data obtained from NMR experiments into the following equations:

$$\frac{1}{T_{2\text{obs}}} = \pi \Delta v_{1/2} = k_{\text{ex}} + \frac{1}{T_{2d}}$$

(1)
\[
\ln (k_{ex}) = - \frac{E_{ex}}{RT} + C_{ex}
\]

(2)

\[
\ln \left( \frac{1}{T_{2d}} \right) = - \frac{E_d}{RT} + C_d
\]

(3)

\[
\ln \left( \frac{1}{T_{2obs}} \right) = \ln \left\{ \exp \left( - \frac{E_{ex}}{RT} + C_{ex} \right) + \exp \left( - \frac{E_d}{RT} + C_d \right) \right\}
\]

(4)

In Eq. (1), \(1/T_{2obs}\) is the observed transverse relaxation rate, \(\Delta v_{1/2}\) is the line width, \(k_{ex}\) is the exchange rate constant and \(1/T_{2d}\) is the dipolar contribution to the line width. In Eq. 2 and 3, \(E_{ex}\) and \(E_d\) are the activation energies; \(C_{ex}\) and \(C_d\) are the Arrhenius coefficients. \(k_{intrinsic}\) was calculated using the equation:

\[
k_{intrinsic} = \sum_{i} \left( \frac{k_D [catalyst], 10^{\Delta pKa}}{10^{\Delta pKa} + 1} \right)
\]

Where \(k_D\), the second order rate constant is \(\sim 10^{10} \text{ M}^{-1}\) at 25 °C, and \(\Delta pKa\) is the difference in \(pKa\) of the proton donor(s) and acceptor(s).

C.2 Method

Since temperature is a variable in this experiment, the sample temperature in the NMR probe has to be determined accurately. A calibration curve (Figure 2.1) was
constructed using neat methanol as a standard. The actual sample temperature was given by the following equation:

\[ T (K) = 403.0 - 29.48 |\Delta \delta| - 23.81 (\Delta \delta)^2 \]

Where \( \Delta \delta \) is the chemical shift difference between methyl and hydroxyl proton of methanol in ppm.

Proton exchange rate with solvent was determined based on the temperature dependence of the width of the corresponding resonance in 1-D \(^1\)H NMR spectra. The sample was equilibrated in the probe for at least 15 min before each acquisition at different temperatures. The line widths of the proton resonance of interest was determined by measuring the width of the peak at half-height in Hz.

(D) Free energy formation of the hydrogen bond

D.1 Theory

Site-directed mutagenesis is often employed to assess the energetic contribution of an individual hydrogen bond. It is achieved by deleting or substituting the hydrogen donor. Estimates obtained from this method generally are < 5 kcal/mol, but there have been reports showing a substantially larger number. Nonetheless, it is noteworthy that these values are the upper limits of energetic contribution by a hydrogen bond itself because in general hydrogen bonds can not be removed without changing other physiochemical properties (e.g. the pKa, dipole moment, etc.) of the protein and the environment in which is hydrogen bond is located.
Figure 2.1 Temperature calibration experiment. The temperature of the NMR spectrometer probe was calibrated in order to obtain accurate temperature measurements for the temperature experiments to determine protection factor.
Figure 2.1

\[ y = 1.497x - 151.2 \]

\[ R^2 = 0.9990 \]
CHAPTER 3

NMR STUDIES OF BOVINE PANCREATIC SECRETED PHOSPHOLIPASE A2 INDICATE THE PRESENCE OF A HYDROGEN BOND WITH LOW BARRIER CHARACTERISTICS BUT WITH RELATIVELY LOW BOND STRENGTH

Introduction

Secreted phospholipases A2 (hereafter designated sPLA2) are small (13 - 14 kDa) calcium-dependent lipolytic enzymes that belong to a family of enzymes that catalyze the hydrolysis of phospholipids at the sn-2 ester bond (Verheij, 1995). It was observed from crystal structures that the active site of sPLA2 is composed of an aspartate-histidine dyad, which couples with the catalytic water molecule to form a catalytic triad analogous (Figure 3.1) to the well-studied serine proteases and lipases. One notable exception is about the orientation of the imidazole ring in the sPLA2 and serine proteases/lipases are flipped with respect to each other. In serine proteases, the catalytic triad consists of Asp, His, and Ser residues; and a proposed low barrier hydrogen bond (LBHB) is formed between Asp and His in both free and complexed states (Cassidy et al., 1997; Lin et al., 1998; Neidhart et al., 2001). Since sPLA2 also employs the general base mechanism, it would be interesting to know whether such a “LBHB” exists and plays an equivalent role. A member of the sPLA2s, bovine pancreatic sPLA2 has been extensively studied by

42
Figure 3.1  (1) Asp-His catalytic dyad and the proposed transition state: (2) The phosphonate transition state analogue. HK32 was designed to mimic the tetrahedral intermediate of sPLA$_2$s catalysis.
Postulated interactions of the catalytic residues of PLA$_2$ with the catalytic water molecule and substrate carbonyl group during the transition state. The ----- line represents the LBHB formed during transition state.

Postulated interaction of the catalytic residues of bovine pancreatic PLA$_2$ with the transition state analogue, HK32. The --- line represents the LBHB detected by NMR.

**Figure 3.1**

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multifaceted approaches including site-directed mutagenesis, scooting-mode kinetics, X-ray crystallography, and nuclear magnetic resonance (NMR) spectroscopy (Yuan & Tsai, 1999). These proteins thus serve as excellent models for investigating such a question.

Here NMR spectroscopy has been employed as a probe for the detection and characterization of a possible LBHB in bovine pancreatic sPLA2. Although the most direct evidence for the existence of a LBHB are the measurement of bond strength and short donor-acceptor atomic distance, the most feasible way to characterize LBHBs in solution has been the observation of several physicochemical properties from NMR spectroscopy. The widely accepted features that are commonly associated with LBHBs are: (i) unusual downfield proton chemical shift, (ii) fractionation factor < 1.0, (iii) exchange rate protection factor > 10, and (iv) hydrogen bond strength ≤ (-7) kcal/mol (Table 3.1; Hibbert and Emsley, 1990; Frey et al., 1994; Mildvan et al., 1999). The distinctive feature is the appearance of a 16 to 20 ppm proton resonance that has been claimed to be the most unambiguous evidence of the presence of a LBHB. Our results demonstrate that bovine pancreatic sPLA2 displays downfield proton resonances. More extensive characterization of the bovine pancreatic sPLA2 shows that three out four of the above criteria are observed. Histidine 15N-specific labeling experiments show that the strong H-bond is formed on the N61 side of the catalytic histidine-48, and presumably with the transition state inhibitor, instead of between the catalytic aspartate-99 and histidine-48 as in the case of many serine proteases. However, the "suggestive LBHB" shows a hydrogen bond strength of merely -4.6 kcal/mol.
1. Proton chemical shift: 15 to 20 ppm,
\[ \Delta \delta = \delta_{\text{observed}} - \delta_{\text{intrinsic}} \geq 5 \text{ ppm} \]

2. Fractionation factor: \( \phi = \frac{[\text{Enzyme-D}][\text{H}_{\text{solvent}}]}{[\text{Enzyme-H}][\text{D}_{\text{solvent}}]} \leq 0.4 \)

3. Exchange rate protection factor: \( \frac{k_{\text{intrinsic}}}{k_{\text{ex}}} \geq 10 \)

4. Hydrogen bond strength \( \leq (-7) \text{ kcal/mol} \)

Table 3.1 NMR criteria for low-barrier hydrogen bonds
RESULTS AND DISCUSSION

Characterization of the downfield proton resonance of the bovine pancreatic sPLA2-HK32 complex

For free bovine pancreatic wild type sPLA2, a downfield peak (≥ 16 ppm) was neither observed in the temperature range of 278-310 K, pH range of 4 – 8, nor in the presence of deuterated DMSO alone. An unusual downfield peak was observed at 17.8 ± 0.1 ppm in the wild type sPLA2-HK32 complex, which signified the presence of a deshielded proton. Peaks shift in one-dimensional $^1$H proton NMR and two-dimensional $^1$H-$^15$N HSQC were also observed upon the addition of HK32, which could be associated with the binding of inhibitor. This proton resonance also exhibited the following physicochemical properties characteristic of a LBHB: (i) The $D/H$ fractionation factor was determined by integrating the down field proton resonance as a function of the $H_2O/D_2O$ solvent ratio. The results (figure 3.2) show that the wild type sPLA2-HK32 complex displays a fractionation factor of 0.56 ± 0.06, indicating a stronger hydrogen bonding relative to a regular hydrogen bond which has typical fractionation factors of approximately 1 for N- and O-bonded protons in water (Perrin and Nielson, 1997). (ii) Upon fitting NMR data to the Arrhenius plot of the effect of temperature on the observed transverse relaxation rate ($1/T_{2obs}$) of the downfield proton resonance in the wild-type sPLA2-HK32 complex, we obtained $k_{ex} = 62$ s$^{-1}$ at $T = 8.5$ °C and $= 236$ s$^{-1}$ at $T = 25.0$ °C. $k_{intrinsic}$ was estimated to be approximately $10^4$ at pH 6.0, yielding an exchange rate protection factor of >100 at $T = 8.5$ °C, and >40 at $T = 25.0$ °C (figure 3.3).
Figure 3.2 Determination of fractionation factor, $\phi$ of the downfield proton resonance in bovine pancreatic sPLA$_2$ and transition-state analogue (HK32) complex. $\phi = 0.56 \pm 0.06$.

$X =$ mole fraction of H$_2$O in D$_2$O.
Equation:
\[
\frac{1}{I} = \phi \left( \frac{1-X}{X} \right) + 1
\]

Figure 3.2
Figure 3.3 Determination of $k_{er}$. Arrhenius plot of the effect of temperature on the observed transverse relaxation rate ($1/T_{2obs}$) of the downfield proton resonance in wild type sPLA$_2$/HK32 complex. $E_{ct} = 13.5 \pm 0.6$ kcal/mol; $C_{ct} = 28.3 \pm 1.0$; $E_d = -4.8 \pm 0.4$ kcal/mol; and $C_d = -3.2 \pm 0.8$. 

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Figure 3.3
Assignment of the downfield proton resonance

For serine proteases (Asp-His-Ser catalytic triad) and a recently reported bacterial phosphatidylinositol-specific phospholipase C (PI-PLC; Asp-His catalytic dyad), the low-field $^1H$ NMR signal (16 – 19 ppm) has been attributed to the proton that is engaged in the hydrogen bond between the $\beta$ carboxylate of Asp and the imidazole $N^\delta1$ of His of the active site (Cleland et al., 1994; Frey et al., 1994; Ryan et al., 2001). It was thus tempting to assign the downfield peak to $H^\delta2$ of H48 bridging His48 and Asp99 in the case of bovine pancreatic sPLA$_2$ (orientation of the imidazole ring is flipped in sPLA$_2$). The down-field proton resonance was not observed in the temperature range of 278-310 K and the pH range of 4 – 8 in free sPLA$_2$, unlike in the case of some serine proteases and PI-PLC. This suggests that the downfield resonance is a unique property of the complex of sPLA$_2$ with HK32. In order to rigorously establish the assignment, a series of NMR experiments were carried out. (1) The two most downfield peaks (17.8 and 12.5 ppm, respectively) were both nitrogen-attached protons based on observation of a $^{15}$N NMR splitting in jump-return experiments carried out on a uniformly $^{15}$N-labeled wild type-HK32 complex sample. The $^{15}$N chemical shifts were determined to be 195 ± 2 and 171 ± 2 ppm, respectively, by performing a series of continuous wave decoupling experiments with different $^{15}$N carrier frequencies. Based on $^{15}$N chemical shifts, it is reasonable to conclude that both peaks originate from the imidazole ring of a histidine residue (Bachovchin, 1986). (2) Since bovine pancreatic sPLA$_2$ only contains 2 histidine residues, H48 and H115, the assignment was simplified by constructing the H115A mutant. Both 17.8 and 12.5 ppm peaks are retained in this mutant upon the addition of inhibitor with almost identical chemical shifts as for the wild type-HK32 complex.
Consequently, the two peaks were assigned to proton(s) attached to the imidazole ring of H48. (3) Jump-return experiments were performed on a $^{15}$N$_{e2}$histidine-labeled wild type sPLA$_2$ to distinguish NH$_{e2}$/H48 from NH$_{61}$/H48. In the absence of HK32, only one cross-peak was observed in the HSQC spectrum at the position of 11.48 ppm in the $^1$H-dimension and 167.8 ppm in the $^{15}$N-dimension. This peak was assigned earlier to NH$_{e2}$/H48 based on its NOE peaks to H$_{e1}$/H48 and H$_{62}$/H48 in 2D NOESY (data not shown). Upon addition of HK32, surprisingly, the peak shifted to 12.5 ppm rather than to 17.9 ppm, and only the 12.5 ppm peak showed a doublet feature. Taken together the peak at 12.5 ppm is conclusively assigned to NH$_{e2}$/H48, while the downfield peak at 17.8 ppm to NH$_{61}$/H48 (figure 3.4).

This surprising yet interesting discovery that the downfield proton is not the one showed by the active site Asp and His residues prompts us to question the assignment reliability of the downfield proton resonance observed in other systems, which are solely based on site-directed mutagenesis experiments or even simple analogous comparison with serine proteases (Viragh et al., 2000; Ryan et al., 2001). In our case for example, initial NMR experiments using the H48Q and D99N mutants revealed the absence of the downfield peak in protein-transition-state inhibitor complex. Furthermore, it has been reported that non-catalytic histidine imidazole ring-attached protons are engaged in strong hydrogen bonding, which also exhibit downfield proton resonances (Kahyaoglu et al., 1997). Conclusive assignment of the downfield proton resonance is absolutely crucial for the interpretation of relevant experimental results.
Figure 3.4  Downfield region of $^1$H NMR spectra at 600 MHz recorded with jump-return pulse sequence on wild type and H115A bovine pancreatic sPLA$_2$ proteins in the presence of HK32 transition-state analogue.  a = imidazole NH$_e$ of H48; b = imidazolium NH$_e$ of H48; and c = NH$_e$ of H48 in the presence of HK32.
Figure 3.4

- U\(^{15}\)N WT, pH 5.2, decoupled at \(\delta_{15N} = 168\) ppm
- U\(^{15}\)N WT, pH 5.2
- \(^{15}\)N\(_{e2}/H48\) WT, pH 7.1, with HK32, decoupled at \(\delta_{15N} = 194\) ppm
- \(^{15}\)N\(_{e2}/H48\) WT, pH 7.1, with HK32, decoupled at \(\delta_{15N} = 171\) ppm
- \(^{15}\)N\(_{e2}/H48\) WT, pH 7.1
- U\(^{15}\)N H115A, pH 7.5, with HK32, decoupled at \(\delta_{15N} = 194\) ppm
- U\(^{15}\)N H115A, pH 7.5, with HK32, decoupled at \(\delta_{15N} = 171\) ppm
- U\(^{15}\)N H115A, pH 7.5, with HK32
- U\(^{15}\)N WT, pH 7.1, with HK32, decoupled at \(\delta_{15N} = 194\) ppm
- U\(^{15}\)N WT, pH 7.1, with HK32, decoupled at \(\delta_{15N} = 171\) ppm
- U\(^{15}\)N WT, pH 7.1, with HK32
- control, WT, pH 6.9, DMSO
- control, WT, pH 6.9

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A possible LBHB between H48 and inhibitor

sPLA₂ joins a growing list of enzymes that display an unusual low field proton NMR signal as well as other physicochemical properties that have been proposed to be characteristic of LBHBs. Both NMR studies (Yuan et al., 1999) and the most recent 0.97 Å ultra-high resolution crystal structure of free bovine pancreatic sPLA₂ (Steiner et al., 2001) show that H48 and D99 form a H-bond involving the $N^t2$ of the imidazole ring. One of the possibilities to explain our NMR results is that H48’s imidazole ring rotates 180 degree around the $C'^1-C'^9$ bond upon addition of the inhibitor. However such a possibility is unlikely for the following reasons: (i) X-ray structures of several sPLA₂-inhibitor (transition state analogue or substrate-derived) complexes do not reveal imidazole ring flipping (Scott et al., 1990; Sekar et al., 1997b; Sekar et al., 1998); (ii) H48 ring flipping would require significant backbone conformational adjustment to bring the $N^61$ of H48 to within hydrogen bonding distance of $O^51$ of D99. Furthermore, note that a simple single bond rotation around the $C_\beta$ and $C_\gamma$ of H48 does not interchange $N^61$ and $N^t2$. The distances are 2.81 and 3.84 Å, respectively, for atomic pairs of $O^51/D99 - N^t2/H48$ and $O^51/D99 - C^52/H48$ in the free state. Therefore the most likely possibility is the formation of a LBHB between $N^61/H48$ and the non-bridged phosphonate oxygen in the transition state analogue. The crystal structures of two bovine pancreatic sPLA₂-transition state analogue complex and bovine pancreatic sPLA₂-inhibitor complex reveal a bond length of 2.62 Å and 2.81 Å, respectively, between $N^61/H48$ and the phosphonate oxygen (Sekar, et al., 1997b; Sekar, et al., 1998). Since the structures give a root-mean-square (r.m.s.) deviation for all backbone atoms of 0.32 Å and 0.24 Å, respectively, the
bond length could well fit into the category of a LBHB, which is generally defined as $2.5 < d < 2.6$ Å (Mildvan et al., 1999). Although the formation of a LBHB between the Asp$^{102}$ β carboxylate and the imidazole N$^{61}$ of His57 (chymotrypsin numbering) in serine proteases is more well-known, the presence of a LBHB between a catalytic residue(s) and substrate/inhibitor has also been widely reported (Usher, et al., 1994; Ramaswamy, et al., 1999; Thoden, et al., 2000).

**Estimation of the hydrogen bond strength**

We did not observe the downfield proton resonance in D99N/HK32 complex, suggesting the absence of LBHB in this mutant. Interestingly, crystal structure of the mutant showed that the carbonyl group of N99 side chain remains H-bonded to N$^{ε2}$ of His 48 as in wild type sPLA$_2$, therefore maintaining the tautomeric form of H48 (Kumar et al., 1994). Apparently, the “exact” positioning and/or the electronic effects of D99 in the catalytic core are crucial for the formation of the LBHB. An estimation of the LBHB contribution to sPLA$_2$ catalysis has been evaluated using the D99N mutant. From previous kinetic studies, substitution of D99 with Asn only resulted in a $10^3$ decrease of $k_{cat}/K_m$, and merely a 20-fold decrease in $k_{cat}$ relative to wild type sPLA$_2$ (Kumar et al., 1994). The latter corresponds only to a $= 1.7$ kcal/mol loss in transition state binding energy.

Apparently this contribution is far less than what should be expected from a LBHB. It has been claimed in a current review article that LBHBs are formed in all enzymatic reactions that involve general-acid or general-base catalysis (Cleland, 2000). The author postulated that the contribution in rate acceleration from a LBHB is worth as much as 5
orders of magnitude, as observed in chymotrypsin for example. This discrepancy
between our data and the published data is addressed later in this paper.

The pKa of H48 was obtained from pH titration experiments using $^{13}$C$_{6}$-histidine-
labeled wild type protein as described (Cassidy et al., 1997). pKa values of ~5.6 and ~
9.0 were obtained for free and HK32 inhibitor-bound wild type bovine pancreatic sPLA$_{2}$,
respectively (figure 3.5A, B). An increment of 3.4 pH units corresponds to a hydrogen
bond strength of ~4.6 kcal/mol. Regardless if the $\Delta G_{\text{formation}}$ of the hydrogen bond is -
1.7 kcal/mol or -4.6 kcal/mol, it is still much lower than the proposed -10 to -20
kcal/mol. This finding leads us to the center of the controversy, which is well described
in Guthrie's review (Guthrie, 1996):

FHF has a strong hydrogen bond (in gas phase);
FHF has a short hydrogen bond (in solid phase);
Therefore, all short hydrogen bonds are strong. (In fact, FHF has relatively weak
H-bond in aqueous solution, -0.82 kcal/mol)

Short hydrogen bonds (in solid state) are strong (in solution):
Systems with short hydrogen bonds have unusual isotopic fractionation factors,
unusual $^1$H-NMR chemical shifts and unusual IR vibration frequencies:
Therefore, any system with any of these unusual properties has a strong
hydrogen bond.

Indeed, in many instances only one or a few of the "standard" criteria of a LBHB are
observed/reported in an individual system. On the other hand, it is still controversial
whether LBHBs satisfy all of the four criteria stated in the "Introduction" section of this
paper. For example, Warshel et al. (Warshel et al., 1995; Warshel & Papazyan, 1996)
have argued that evidence for short bond lengths of LBHBs in enzymes does not imply
Figure 3.5  Determination of pKa of H48 in the A. free state and B. complex. pKas of H48 are 5.6 and 9.0, respectively.
Figure 3.5

(continued)
Figure 3.5 continued

B.
an unusually strong bond nor does the presence of an LBHB mean that it has an important role in catalysis.

The pH titration experiment using $^{15}$N$_{e}$-histidine-labeled wild type protein confirmed that H48 is an imidazolium species (positively charged) in the presence of the transition state analogue (Figure 3.6). These results are in agreement with the proposed mechanism by Scott et al. (1990), in which the catalytic histidine residue is a positively charged species in the transition state.

The pKa of H115 was also determined by the pH titration experiment using $^{13}$C$_{e}$ histidine-labeled wild type protein, and was determined to be $\sim 6.1$ (Figure 3.7). At extreme pHs ($< 3$), the chemical shifts of the peak exhibited the value of a random coil histidine. This is consistent with the solution structure of the enzyme, in which the C-terminus is considered a relatively flexible region. At low pH the C-terminus becomes unstructured, and therefore, H115 exhibited the value of a random coil histidine (Figure 3.8).

A possible role of LBHB in sPLA$_{2}$

The implication of a LBHB between sPLA$_{2}$ and the transition-state analogue on the mechanism of sPLA$_{2}$ catalysis can be envisioned. A mechanism for sPLA$_{2}$ catalysis was proposed in 1980 based on structural comparison with the serine proteases (Verheij et al., 1980). The reaction pathway involves general base-catalyzed attack of a conserved water molecule onto the carbonyl carbon of the sn-2 ester. The structural features of this proposal have been confirmed by X-ray studies with transition-state and substrate analogues for three of the four sPLA$_{2}$ classes (Scott et al., 1990). Since the down-field
Figure 3.6  HSQC spectra of WT bovine pancreatic sPLA₂.  A. A portion of the HSQC spectrum of ¹⁵N-uniform label protein at pH 5.2. The peak was previously assigned to H48 imidazole ring. Since the pKa of H48 in free PLA₂ is ~5.6, the chemical shift of the peak represents a neutral imidazole species.  B. Spectrum of ¹⁵Nₑ/H₄₈ specific label protein at pH 7.1. The chemical shift represents a positively charged imidazolium species.  C. Spectrum of ¹⁵Nₑ/H₄₈ specific label protein in the presence of HK32 at pH 7.1. Since the pKa of H48 in the presence of HK32 is ~9.0, the chemical shift represents a neutral imidazole species.  D-E. Spectrum of ¹⁵Nₑ/H₄₈ specific label protein in the presence of HK32 at pH 8.5 and 9.9, respectively. Both neutral and charged species of the imidazole ring can be observed in D. The chemical shift in E represents a positively charged imidazolium species because the pKa of H48 in the presence of HK32 is determined to be 9.0.
Figure 3.6
Figure 3.7  pH titration experiment of H115 using $^{13}$C$^{15}$N histidine specific-labeled WT protein. The pKa is determined to be ~ 6.1.
Figure 3.7
Figure 3.8  pH titration experiment of H115 at low pH. A gradual shift of chemical shift to a random coil value of histidine is observed on HSQC spectra.
Figure 3.8
proton was not observed in free sPLA₂ in the pH range of 4-8 and temperature range of 278-310 K. but arises upon the addition of the transition-state analogue, stabilization energy released from the formation of a LBHB may stabilize an intermediate or the transition state, and thus lower the activation barrier of esterolysis reaction.

Assuming that the phosphonate transition-state analogue emulates the tetrahedral intermediate during esterolysis, then the non-bridging phosphonate oxygen involved in the LBHB formation represents the attacking hydroxyl resulted from the catalytic histidine’s abstraction of a proton from the conserved catalytic water molecule found in all high-resolution free enzyme crystal structures. During the catalysis, the oxyanion, Ca²⁺ and attacking nucleophile are buried in the enzyme’s interior, where they are devoid of hydrogen bonds to solvent. The LBHB offers a stabilization effect during transition state, but not the ground state, thus reducing the activation barrier of the reaction. Our finding is supported by the fact that amide substrate analogue of sPLA₂ binds most effectively under basic condition, in which the catalytic histidine is neutral. On the contrary, the phosphonate transition-state analogue has greatest affinity to the protonated form of enzyme, in which the catalytic histidine residue carries an imidazolium species (Thunnissen, et al., 1990; Yu and Dennis, 1991).

Comparisons with the serine proteases and other Asp-His catalytic dyads

In a quick glance, serine proteases and sPLA₂s utilize a similar active site architecture and employ a general-base mechanism. Nonetheless, there are major yet intricate differences in terms of the role of LBHBs in catalysis. (i) The site of formation
of the LBHB is different. While the LBHB is formed between the catalytic Asp and His residues in serine proteases, it is between the catalytic histidine residue and an oxygen of the bound transition-state analogue. (ii) The LBHB is absent in inhibitor-free sPLA$_2$, but present in both free and complexed serine proteases. (iii) The magnitude of the contribution of the LBHB could be substantially less in sPLA$_2$, as projected from the proposed hydrogen bond strengths. We may attribute these differences to the fact that perhaps a Asp-His-Ser triad is much different from a Asp-His dyad. Interestingly, it has been reported that the prolyl oligopeptidase family of serine proteases exhibits important dissimilarities in the active site compared to the pancreatic and subtilisin classes of serine proteases. In the case of a prolyl oligopeptidase, LBHB is absent in the free enzyme (Kahyaoglu et al., 1997). More recently, a LBHB was observed between the catalytic aspartate$^{274}$ and histidine$^{32}$ residues of phosphatidylinositol-specific phospholipase C (PI-PLC), which has a Asp-His dyad active site (Ryan et al., 2001). However, note that the imidazole ring in PI-PLC has the same orientation as in serine proteases). The authors also reported other NMR criteria for short, strong hydrogen bond, such as a highly deshielded proton resonance, short bond length, low fractionation factor and large protection factor. However, previous studies on PI-PLC mutants, including D274N mutant only yielded an approximated 60-fold reduction in $k_{cat}$, which corresponding to $\sim$2.4 kcal/mol in energy (Hondal, et al., 1998).

In conclusion, our results show that bovine pancreatic sPLA$_2$ meets 3 out of 4 of the criteria of a LBHB formed between the N$^{e2}$ of H48 and the non-bridging phosphonate oxygen in the transition state analogue. The LBHB may serve as a stabilizing factor in the catalytic core during transition state, thus lowering the activation
energy of the reaction. However the contribution of the stabilizing effect of a LBHB (if applies) is not as significance as in the case of serine proteases in the catalysis of bovine pancreatic sPLA$_2$. 
Part II

SOMATIC INK4a-ARF LOCUS MUTATIONS: A SIGNIFICANT MECHANISM OF GENE INACTIVATION IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS
CHAPTER 4

INTRODUCTION

The cell cycle

A cell constantly receives extrinsic, as well as intrinsic signals (mitogenic and anti-mitogenic) that govern its fate, that is either to stay quiescent, to divide, or to die. Cell-cycle checkpoints are absolutely crucial for normal cellular processes of proliferation, differentiation and programmed cell death (apoptosis). Defects in cell-cycle checkpoints are often associated with a broad spectrum of phenotypes, including cancer predisposition and neuro-degeneration, establishing the fact that checkpoint responses are the deciding factors for cell survival or cell death (figure 4.1). The two most important checkpoints are the G1/S and G2/M phase transition. The G1 checkpoint, which is commonly referred to as “Start” in yeast and “Restriction point” in mammalian cells, is the point at which a cell decides to commit to DNA replication and complete a full cycle of cell division (Hartwell et al., 1974; Pardee, 1974). A family of serine-threonine protein kinases, named the cyclin-dependent kinases (CDKs) regulates the temporal order and passage through each checkpoint (Figure 4.2a). In eukaryotes, there are numerous different CDKs acting on different phases of the cell cycle. CDKs are regulated in several different ways, including the binding of activating cyclin subunits.
Figure 4.1  Checkpoints control the progression of the cell cycle by determining if the preceding phases have been completed flawlessly.
Figure 4.1
inhibition of Cip or INK4 proteins, phosphorylation, dephosphorylation, and ubiquitin-mediated degradation to yield different downstream outcomes.

To date, 9 CDKs and 16 cyclins have been identified in mammalian cells (Johnson & Walker, 1999). It should be pointed out that not all CDKs and cyclins are involved in cell cycle regulation. Functions of specific CDKs and cyclins include DNA repair, differentiation, and etc. (Roy et al., 1994; Rickert et al., 1996). Monomeric CDK is inactive, and its complete activation is accomplished in two steps. First, binding to a cyclin subunit yields a partially active CDK heterodimer. Then, phosphorylation of the heterodimer by a CDK-activating kinase, CAK fully activates the complex (Fisher & Morgan, 1994; Fisher, et al., 1995). Two families of CDKs inhibitory proteins, Cip (CDK inhibitory proteins) and INK4 (Inhibitors of CDK4) proteins can counteract the monomeric and heteromeric forms of CDKs. It has been found that INK4 proteins bind and inhibit CDK 4 or 6 monomer and the unphosphorylated cyclin-CDK complex. In addition to the above, the Cip proteins also bind and inhibit the phosphorylated cyclin-CDK (Figure 4.2b; reviewed by Sherr & Roberts. 1995; reviewed by Serrano. 1997).

Proteins that are key players in checkpoint control are often the targets of genetic alterations in cancer because of their critical role in cell proliferation (Sherr. 1996). Malignant transformation is often related to genetic aberrations, which give rise to oncogenes with dominant gain-of-function (Aaronson, 1991; Hirota et al., 1998), or tumor suppressor genes with recessive loss-of-function (Weinberg. 1991; Campiglio et al., 1999). Since inactivation of both copies of a tumor suppressor gene is required for loss of function, in other words heterozygosity at the locus is phenotypically normal. loss

77
Figure 4.2  

a. Proteins shaded in red are found implicated in cancer.  
b. Flowchart of the general regulatory mechanism of CDKs. CAK is the abbreviation for Cdk-activating kinase; encircled P represents a phosphate group. Arrows represent positive regulation, while bars represent negative regulation.
Figure 4.2 (continued)
Figure 4.2 continued

b.

CDKs
inactive

INK4 inhibitors
conformational change

Cip inhibitors

partially active

conformational change

Fully active
of heterozygosity (LOH) is a common genetic aberration in tumor suppressor genes (Murthy & Testa, 1999; Rasheed et al., 1999; Velickovic et al., 2001). In terms of pathogenesis of cancer, cell cycle defects can either be a predisposing event, for example germ-line mutations, or contribute to the progression of tumorigenesis and malignant transformation as the results of additional somatic mutations (Hartwell & Kastan, 1994; Strauss, et al., 1995; Elledge, 1996; Hall & Peters, 1996; Sherr, 1996; Fearon, 1997).

The INK4 family

To date, there are only four known members of the INK4 family, and they are p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, p18\textsuperscript{INK4C}, and p19\textsuperscript{INK4D} (hereafter referred to as p16, p15, p18 and p19, respectively). p16 and p15 are located tandemly on human chromosome 9p21 (Hannon & Beach, 1995), while p18 and p19 are on human chromosome 1p32 and 19p13.2, respectively (Camero & Hannon, 1997). The INK4 proteins share some sequence homology, however, p16 is more closely related to p15 (about 85\% sequence homology), while p18 is closer to p19 (about 40\% sequence homology). The INK4 proteins specifically interact with CDK4 and CDK6 but not other CDKs. Upon binding to CDK4 or 6, they prevent the association of the CDK with cyclin D. However, INK4 proteins are still able to inhibit the pre-assembled CDK4/6-cyclin D complex.

The Role of p16\textsuperscript{INK4a} (MTS1/CDKN2)

The existence of p16 was first noticed in human diploid fibroblasts transformed by SV40 virus. It was noted that CDK4 predominantly associated with a 16 kDa protein.
rather than cyclin D (Xiong et al., 1993). p16 was first identified by yeast-two-hybrid screening using CDK4 as the bait. Immunoprecipitation experiments showed that p16 binds to CDK4 and CDK6, but not CDK2, CDK5, cdc2 or PCNA (Serrano et al., 1993). The full-length gene was discovered two years later, which encoded a polypeptide of 156 amino acid residues (Quelle, et al., 1995). The INK4a locus located on human chromosome 9p21 has been found to be a hot spot for loss of heterozygosity (LOH) and homozygous deletion in a variety of human cancers (Kamb et al., 1994; Ruas & Peters, 1998). Genetic alterations in the p16 gene, including deletion, point mutation, and hypermethyllation silencing have been found in at least one third of all human cancers (Serrano et al., 1993; Kamb et al., 1994; Nobori et al., 1994). p16 plays a critical role in regulating the status of pRb in cell. Cells deficient in pRb protein show an elevated level of p16 protein due to the disruption of a feedback loop in which pRb represses p16 gene expression (Li et al., 1994).

p16 protein belongs to the ankyrin-repeat-containing protein family. It is comprised of four tandemly repeated ankyrin motif (helix-turn-helix motif). The p16 solution structure as well as the p16/CDK6 complex crystal structure were determined in 1998 (figure 4.3; Tevelev et al., 1996; Byeon, et al., 1998; Russo et al., 1998). The p16/CDK6 complex crystal structure reveals that p16 binds next to the catalytic cleft of CDK6, interacting with both the N- and C-termini of the kinase. This may explain why p16 can bind to the pre-assembled CDK 6/cyclin D complex without dissociating cyclin D. Essentially, p16 is an allosteric inhibitor of CDK4 and 6. The complex structure shows that p16 inhibits the CDK/cyclin complex by disrupting the optimal interaction.
Figure 4.3 A ribbon diagram of the p16\textsuperscript{INK4a} solution structure. Restrained minimized mean structure. PDB 2A5E (Byeon, et al., 1998)
between the kinase and cyclin for efficient catalysis. Cross-linking experiment using an ATP analog demonstrated that the binding of p16 weakens ATP affinity of the kinase. A close-up look at the structure of the kinase/cyclin complex indeed reveals a distorted ATP binding site (Russo et al., 1998).

Normal somatic cells only proliferate a limited number of times, generally 20 to 30 doubling times before entering a permanent growth arrest stage, referred to as senescence, in which they remain metabolically active but insensitive to mitogenic stimuli (Hayflick & Moorhead, 1961). Experimental results suggest that senescence may be a tumor suppression mechanism (Sager, 1991; Campisi, 1997). It has been noticed that the expression level of p16 protein is elevated in cells engaged in senescence (Alcorta, et al., 1996; Palmero, et al., 1997; Erickson, et al., 1998). Meanwhile, many reports showed that the function of p16 is lost or impaired in immortalized cells (Okamoto, et al., 1994; Brenner & Aldaz, 1995; Noble, et al., 1996; Vogt, et al., 1998).

**One locus, two pathways**

Understanding of the cellular function of p16 is complicated by the fact that the INK4a locus also encodes another gene, ARF (p19ARF in mouse, p14ARF in human). p16 transcript contains the exons 1α, 2, and 3; whereas ARF transcript contains the exons 1β, 2, and 3 (Figure 4.4). These genes are regulated by their own promoter (Haber, 1997). Although these genes share exon 2 and 3, the proteins translated are distinct due to different reading frame. Both p16 and p19ARF (hereafter ARF) are tumor suppressors, but each is involved in the cell cycle control via different regulatory pathway. p16 is a key
Figure 4.4 Genomic structure of the INK4a/ARF locus. This structure is conserved in human, mouse and rat.
Figure 4.4
component in the pRb pathway, as described above; while ARF plays a crucial role in the p53 pathway by binding to MDM2. MDM2 is a negative feedback regulator that terminates the response of p53 by abolishing its transactivation ability, and subsequently sends p53 to the ubiquitin degradation pathway. ARF binds and inhibits MDM2, thus stabilizes and activates p53 tumor suppressor, which halts the cell cycle in response to unfavorable stimuli (Pomerantz. et al.. 1998; Zhang, et al.. 1998; Kamijo, et al.. 1998).

Since p16 and ARF share the same locus, thus most mutations can affect both genes. However, certain genetic alterations occur solely on either exon 1α or 1β, therefore impairing the function of one gene product but not the other. This enables us to distinguish the biological function of each protein in a separate manner. For example, certain point mutations found solely on exon 1α are inherited in melanoma kindreds, correlating the function of p16, but not ARF in the genesis of melanoma (Kamb. et al.. 1994; Gruis, et al.., 1995). Certain point mutations in exon 2, which are predicted to alter both p16 and ARF have been investigated. It has been found that often the function of the mutant p16 proteins are impaired without affecting the ability of ARF to induce growth arrest (Quelle et al.. 1997).

The pRb and p53 pathways

During the G1 phase of the cell cycle, pRb is in hypophosphorylated state and active. It binds to several transcriptional factors of the E2F family, and therefore represses the transcription of the downfield genes, which products are required for the entry into S phase (Dyson, 1998). It has been found that E2F transcription factors
regulate the expression of proteins that are involved in cell cycle progression, such as cyclin E, CDK 1, DNA polymerase α, and etc. (Botz et al., 1996; Yamaguchi et al., 1997; Dalton, 1992). Before a cell enters S phase, cyclin D-dependent kinases, CDK4 and/or CDK 6 phosphorylate pRb, thus inactivating the protein. This in turn enables the release of E2F transcription factors from pRb, and subsequently activates a series of downfield genes. As potent inhibitors for CDK 4 and CDK 6, the INK4 proteins can prevent cells with functional pRb from entering S phase. Therefore, defect in the p16-cyclin D-CDK4/6-pRb pathway has been found a common incident in various cancers. Another very important finding is that the assembly of CDK 4/6 and cyclin D requires a member of the Cip/Kip family (p21, p27, and p57). Therefore, the formation of CDK 4/6-cyclin D complex facilitate the G1 to S phase transition in 2 ways: First, by inactivating pRb via phosphorylation, which releases the E2F transcription factors, and by sequesting the Cip/Kip inhibitors, which in turn facilitate the CDK 2-cyclin E-mediated pRb hyperphosphorylation. The consequence of pRb hyperphosphorylation is the further release of transcription factors, which are essential for S phase entry (Harbour et al., 1999). Therefore, the binding of the INK4 proteins to CDK 4/6 not only inhibits the kinase activity via destabilization of cyclin Ds, but also re-distributes the Cip/Kip proteins to CDK 2/cyclin E, thus further inhibits G1 progression (Sherr & Roberts, 1999).

The transcription factor p53 has been found to inhibit cell cycle progression or induce apoptosis in response to specific stimuli (Giaccia & Kastan, 1998). To date, p53 has been shown to be the most inactivated gene in human cancers, in which > 50 % of human cancers contain mutation(s) in this gene (Hollstein, et al., 1994). Mutations in p53 desensitize the cells to cell cycle arrest, as well as to DNA-induced apoptosis, which
often lead to genomic abnormalities, and uncontrollable cell proliferation (Kastan et al., 1991; Kuerbitz et al., 1992; Livingston et al., 1992; Clarke et al., 1993; Fukusawa et al., 1996; Jacks & Weinberg, 1996; Paulovich et al., 1997; Gualberto et al., 1998). Various reports suggest that the pRb and p53 pathways be somehow connected. For example, Loss of function of pRb can bypass p53-mediated G1 arrest (Demers, et al., 1994), and induce E2F and p53-dependent apoptosis (Morgenbesser, et al., 1994; Qin, et al., 1994; Symonds, et al., 1994; Wu & Levine, 1994). These results suggest that defect in the p16-cyclin D-CDK4/6-pRb pathway may be counterbalanced by the p53-dependent apoptosis in order to eliminate cells that derail from the normal cell cycle (figure 4.5). While the INK4 proteins are the CDK inhibitors in the pRb pathway, the Cip/Kip proteins plays inhibitory role in the p53 pathway. The Cip/Kip proteins include p21^{Cip1} (p21), p27^{Kip1} (p27), and p57^{Kip2} (p57). It has been shown that the p21 gene is activated by p53 in response to DNA damage. Induction of p21 regulates the cell cycle in two ways. I) by inhibiting numerous cyclin/CDK complexes, and II) by inhibiting DNA synthesis via p21 binding to PCNA, an elongation factor for DNA polymerase δ, as well as a component of the DNA repair machinery (Li et al., 1994). Similar to the case of p21, p27 and p57 bind to a variety of CDK/cyclin complexes. Nonetheless, p57 demonstrates tissue-specific expression (Lee et al., 1995).

**Targets of Cancer Therapy**

Since uncontainable cell progression is the hallmark of cancer, cell cycle checkpoints are often the target of new therapeutic approaches in cancer therapy. Much
Figure 4.5 Biochemical connection between p16-cyclin D-CDK4/6-pRb and p53 pathways.
effort has been put forth to develop treatments that block cell cycle progression, and induce growth arrest, which is subsequently followed by apoptosis in transformed cells. Nonetheless, a compromised checkpoint could also be exploited for therapeutic proposes. Cells with aberrant cell cycle checkpoints are more sensitive to genotoxins and DNA-damaging reagent, such as radiation (Hartwell & Kastan, 1994). The central role of the CDKs has prompted tremendous attention in developing specific CDK inhibitors. For the purpose of relevancy of this dissertation, we will limit the discussion to G1 phase and G1/S transition, in particular the pRb pathway. Uncontrollable proliferation arises as the results of either loss of function of the negative regulator(s) (p16 or pRb), or overexpression of the proto-oncogene(s) (cyclin D and CDK4/6) (Ruas & Peters, 1998).

Since pRb is the central player in the pRb pathway, the type of therapeutic strategy employed relies heavily on the status of pRb in the transformed cells. Although loss-of-function in pRb is common in certain types of cancer, most human cancers retain wild type or functional pRb. Amplification of CDK 4, overexpression of cyclin Ds, and p16 inactivation are common events observed in transformed cells with an intact pRb protein. Nonetheless, the events mentioned above are usually mutually exclusive, in other words usually only one of the events is found in a particular transformed cell. Any of the events would lead to similar consequence. In cells that do not retain function pRb protein, however, the therapeutics should be focused on other phases and/or checkpoints of the cell cycle (which will not be discussed here). In transformed cells with intact pRb, down-regulation of CDK 4/6 activity may be a plausible strategy to control aberrant cell growth because CDK 4/6 regulates the activity of pRb. Figure 4.6 illustrates strategies to down-regulate CDK 4/6.
Figure 4.6 Approaches to down-regulate the activity of Cdk 4/6. Down-regulation of cyclin Ds: Antiestrogens and retinoic acid have been found to down-regulate cyclin D1 level in breast cancer (Wilcken, et al., 1997; Kurie, 1999). Rapamycin destabilizes cyclin D1 mRNA and protein (Hashemolhosseini et al., 1998). The Cdk inhibitor, Flavopiridol also found to repress cyclin D1 expression. It is currently in phase II clinical trial used in non-small lung cancer (Carlson et al., 1999; Shapiro et al., 2001). Down-regulation of Cdk 4: The molecular chaperone Hsp 90 binds and stabilizes newly synthesized Cdk 4 for correct folding of the kinase. Geldnamycin binds and inactivate Hsp 90, leading to the down-regulation of Cdk 4 and Cdk 6. (Stepanova et al., 1996; Srethapakdi et al., 2000). Activation of p16: demethylation reagent such as 5-aza 2’doxycytidine, and histone deacetylase inhibitor can cause re-expression of p16 gene, which is silenced by hypermethylation. Short p16-derived peptide has been found to induce G1 arrest in cells with intact pRb. This short peptide can serve as a model for designing small molecule inhibitors. The p16-cyclin D-Cdk 6 complex structure is a useful source for rational drug design (Fahraeus et al., 1998; Russo et al., 1998). Adenovirus-mediated delivery of p16 gene in p16 null cancer cells potently inhibits growth (Jin et al., 1995). Adapted from Shapiro & Harper. 1999.
Down-regulation of Cyclins D:-
- Antiestrogens
- Retinoic acid
- Rapamycin
- Flavopiridol

Activation of p16:-
- Demethylation agent
- Histone deacetylase inhibitor
- p16 derived peptide and analogue
- p16 gene replacement by recombinant adenoviruses

Down-regulation of Cdk :-
- Geldanamycin
- Flavopiridol

Figure 4.6
CHAPTER 5

MATERIALS AND METHODS

Sample Procurement.

One hundred SCCHN tissues and patient-matched normal tissues were obtained from the Tissue Procurement Service at the Ohio State University Comprehensive Cancer Center. The samples were further subdivided into the following site-specific categories: pharynx (32 samples), larynx (24 samples), oral cavity (15 samples), and unclassified (29 samples).

DNA Isolation

Genomic DNA was isolated from either trimmed frozen tissue (78 samples) utilizing TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions or microdissected from paraffin-embedded histological tissue sections (22 samples) using a standard octane/proteinase K extraction procedure (Hongyo. et al., 1995).

PCR Amplification.

Exons 1α, 1β and 2 of p16/ARF gene were amplified using the intron-based primers listed in Table 5.1. Exon 2 was amplified in two fragments (2a and 2b) with at
<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon</th>
<th>Oligonucleotide primer sequences (pair) for PCR and SSCP conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Sequence (5' → 3')</strong></td>
</tr>
<tr>
<td><strong>p16</strong></td>
<td></td>
<td>Fragment size (bp)  SS Cp (<em>a</em>)  SS Cp (<em>b</em>)</td>
</tr>
<tr>
<td></td>
<td>1α</td>
<td>GCT GCG GAG AGG GGG AGA GCA GGC A GCG CTA CCT GAT TCC AAT TC</td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>ACA AGC TTC CTT TCC GTC ATG CCG CCA GGC ATC GCG CAC GTC CA</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>TTC CTG GAC ACG CTG GTG GT TCT GAG CTT TGG AAG CTC TCA G</td>
</tr>
<tr>
<td></td>
<td>1β</td>
<td>AGT GGC GCT GCT CAC CTC TC AAG TGC GCC CCG GAC TTT TC</td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td>ACA AGC TTC CTT TCC GTC ATG CCG CCA GGC ATC GCG CAC GTC CA</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>TTC CTG GAC ACG CTG GTG GT TCT GAG CTT TGG AAG CTC TCA G</td>
</tr>
</tbody>
</table>

* Primer sequences for p16 fragments 1α, 2a, and 2b were described by Zhang, et al., 1994.

**Table 5.1.** Oligonucleotide primer sequences (pair) for PCR and SSCP conditions
least one of the primers in a set located within an intron, to exclude co-amplification of a similar gene family member or a potential pseudogene, as well as to confine the size of the amplified fragment suitable for SSCP analysis. Exon 3 was excluded from our evaluation because it represents only 3% (12 bp) and 0% of the pl6 and pl4 \text{ARF} genes, respectively. The 10 µl PCR mixture was composed of 1x PCR buffer, 1.5 mM MgCl₂, 200 µM of each dNTP, 0.4 U Taq polymerase (Life Technologies) bound to TaqStart Antibody (Clontech, Palo Alto, CA), 2 µM of each primer, 50 ng of DNA template, and 5% DMSO (pl6 exon 2a and 2b) or 3.6% formamide (pl6 exon 1α and 1β). After incubation for 1 min at 94 °C in a thermal cycler (MJ Research, Watertown, MA), all PCR tubes were cycled through: 50 sec at 94 °C, and either 50 s at 60 °C or 50 s at 72 °C for 30-35 cycles, followed by 7 min at 72 °C, and a final 4 °C soak. Verification of stringent PCR conditions was obtained by analyzing the PCR fragments on pre-cast 10% polyacrylamide mini-gels (Novex, San Diego, CA). All reactions were conducted in duplicate to control potential Taq polymerase-induced errors.

"Cold" SSCP Analysis.

SSCP analysis was conducted utilizing a novel, non-radioactive modification of the technique, which is more sensitive and rapid than conventional radioactive SSCP protocols (Hongyo, et al., 1993). In this procedure, 1-5 µl of the PCR-amplified fragments were diluted with either: i) 2 µl of Ficoll loading buffer, 12.6 µl of 1.25x Tris-Borate-EDTA (TBE) buffer, and 0.4 µl of 1M methylmercury hydroxide (Alfa AESAR, Johnson Matthey Catalog Co., Ward Hill, MA) or ii) 0.6 µl of Ficoll loading buffer, 7 µl of formamide, and 0.4 µl of glycerol. Samples were heated at 85 °C for 3 min, placed
immediately on ice, and loaded onto precast 20% polyacrylamide mini-gels (Novex, San Diego, CA). Electrophoresis was conducted in a ThermoFlow SSCP system (Novex, San Diego, CA) at 300 V in 1.25x TBE buffer, which was maintained at a temperature empirically determined to be optimal for identifying several known positive controls in each PCR fragment. Circulating TBE buffer was constantly chilled at unique, optimized temperatures for each fragment using the ThermoFlow SSCP System. The optimized temperatures (Table 5.1) were empirically determined using positive control PCR fragments known to contain single nucleotide substitutions from a previous study (Chen et al., 1996) or generated by site-directed mutagenesis (Sarkar & Sommer, 1990). Gels were stained with SYBR Green II (Molecular Probes, Eugene, OR) and imaged with the IS1000 gel documentation system (Alpha Innotech Corp., San Leandro, CA). Positive SSCP samples were confirmed by analyzing the corresponding duplicate PCR sample and by direct sequencing for the identification of the specific mutation. When a confirmed, positive SSCP mutant band represented less than 50% of the allelic copies due to dilution with wild-type alleles (e.g. infiltrating lymphocytes, etc.), a portion of the mutant band was removed from the polyacrylamide SSCP gel, re-amplified by PCR, re-examined by SSCP to ensure enrichment, and then sequenced.

**Sequence Analysis.**

Direct DNA sequencing was performed on the PCR-amplified DNA fragments to detect mutations within the primary tumors. Following PCR, 50 μl of the PCR product was washed and concentrated to 40 μl using Micron-100 microconcentrators (Amicon, Beverly, MA). Purified PCR product was sequenced on an Applied Biosystem 377.
automated DNA sequencer using the ABI PRISM™ dye terminator cycle sequencing ready reaction kit (ABI/PE, Foster City, CA) according to the manufacturer's protocol. Samples were loaded onto 4.25% denatured polyacrylamide gels (0.2 mm thick), electrophoresed at 3000 V for 3.5 h, and analyzed using ABI sequencing analysis software. All mutations were confirmed by sequencing both DNA strands.

Computer Analysis.

A mutant sequence identified within intron 1 of the p16 gene was analyzed for potential effects on mRNA splicing using the Signal program of the PC/Gene software package (IntelliGenetics, Campbell, CA).

Construction, Expression and Purification of p16 and Mutants.

Several of the unique somatic mutations identified in this study were chosen for protein re-construction and functional analysis [Sample # 63 (53-58Δ mutant); # 132 (58 stop mutant); # 133 (110 stop mutant); # 135 (124 fs mutant); # 156 (L97P mutant); # 321 (D116Y mutant); and # 94-3581 (L63V mutant)]. Human p16 cDNA was cloned into pGEX-2T vectors and expressed as a glutathione S-transferase (GST)-fusion protein in Escherichia coli (E. coli) strain BL 21 (DE 3) pLys S (Novagen, Madison, WI) (Byeon, et al., 1998). All mutants were constructed by the Quickchange method (Stratagene, LaJolla, CA) using pGEX-p16 as template. The cell lysate was centrifuged at 40,000 x g at 4 C for 1 hr, and the supernatant was loaded onto a glutathione-agarose column (Sigma, St. Louis, MO). Thrombin digestion-resistant proteins, including wild-type P16, L63M, L63N, L63V, and D116Y, were cleaved from the column with
thrombin (Sigma, St. Louis, MO), and further purified on a S-100 column equilibrated with 4 mM HEPES buffer containing 1 mM DTT and 5 mM EDTA at pH 7.5. The thrombin digestion-sensitive proteins, such as the truncated mutants, were eluted from the glutathione-agarose column with 50 mM reduced glutathione (pH 7.5), and further analyzed as GST-fusion proteins.

**CDK4 Kinase Assay.**

Evaluation of wild type and mutant P16, utilizing the *in vitro* CDK4 kinase assay, was conducted as previously described (Byeon, *et al.*, 1998). Briefly, 10 units of the CDK4/cyclin D2 complex and varying amounts of P16 in a total volume of 15 µl kinase buffer (50 mM HEPES, 10 mM MgCl₂, 2.5 mM EGTA, 0.1 mM Na₃VO₄, 1mM NaF, 10 mM β-glycerophosphate, 1 mM DTT, 0.2 mM AEBSF, 2.5 mg/ml leupeptin, and 2.5 mg/ml aprotinin at pH 7.5) were mixed and incubated at 30 °C for 30 min. Assays for each mutant P16 were done in triplicate and a sample with null P16 was included as a negative control. Subsequently, 50 µg of GST-pRb and 2 µCi of [γ-³²P] ATP were added into the reaction mixture, the samples were incubated at 30 °C for an additional 15 min, and the reaction stopped by adding 3.75 µl of regular 5x SDS-PAGE loading buffer. The reaction mixture was separated by SDS-PAGE and the incorporation of ³²P was analyzed using a Phosphorimager. The substrate of CDK4, GST-pRb, was prepared as described elsewhere (Byeon, *et al.*, 1998).
Nuclear Magnetic Resonance (NMR) Analysis of p16 L63 Mutant Proteins.

All NMR experiments were performed on a Broker DMX-600 spectrometer at 20 °C (Byeon, et al., 1998). The protein concentration of the NMR samples was 0.2-0.4 mM contained in 4 mM HEPES, 1 mM DTT, and 5 μM EDTA in 100% ²H₂O at pH 7.5.
CHAPTER 6

SOMATIC MUTATIONS SCREENING OF THE INK4a-ARF LOCUS

Introduction

Cancers of the head and neck (HN) represent approximately 4% of the malignant neoplasms diagnosed in the United States (Landis, et al., 1999) and the sixth most prevalent cancer worldwide (Parkin, et al., 1999). The majority of HN cancers are squamous cell carcinomas (SCC), which have been etiologically linked to individual exposure to known carcinogens, such as tobacco and alcohol (Rothman & Keller, 1972; Million, et al., 1989). The overall survivorship of SCCHN is relatively low, with a 50% five-year survival rate for all sites and stages (Shah & Lydiatt, 1995).

Over the past few years, an extensive effort has been underway to identify the molecular events involved in the development of SCCHN. In cancer research, one major focus of research activity has been genes which products are associated with the G1 to S phase checkpoint. Several of these genes, including the p53 tumor suppressor gene (Somer, et al., 1992; Boyle, et al., 1993; Brennan, et al., 1995), and cyclin D1 oncogene (Saranath, et al., 1989; Callender, et al., 1994; Jares, et al., 1994). In recent years, the INK4a/ARF locus has been the interest of many research projects.
Inactivating events at the INK4a/ARF locus, including homozygous deletion, promoter hypermethylation, somatic and germline point mutations, have been reported in a wide variety of cell lines and primary tumors, such as melanomas (Hussussian, et al., 1994; Kamb, et al., 1994), pancreatic adenocarcinomas (Caldas, et al., 1994; Huang, et al., 1996), and esophageal SCC (Mori, et al., 1994; Zhou, et al., 1994), with high frequency. Conversely, in SCCHN the significant inactivation modes reported have been deletions and hypermethylation (González, et al., 1995; Lo, et al., 1996). The incidence of somatic INK4a/ARF alterations in primary SCCHN has been reported to be quite rare (0-10%) (González, et al., 1995; Cairns, et al., 1994; Zhang, et al., 1994; Uzawa, et al., 1995), intimating that somatic alterations in this locus play a minor role in HN cancer. This study examined if somatic mutational inactivation in this locus has been previously underestimated. To address this question, we have performed mutational analysis of both p16 (exons 1α and 2) and p14ARF (exons 1β and 2) in 100 primary SCCHN and patient-matched normal tissues using polymerase chain reaction (PCR), “Cold” single-stranded conformation polymorphism (SSCP) analysis, and direct DNA sequence analyses. Subsequently, representative mutant p16 proteins that are unique to this study were constructed and evaluated in vitro for their functional activity. Structural and functional studies were limited to p16 proteins due to the unavailability of high purity p14ARF protein.
RESULTS

**Mutational Analysis of the INK4a-ARF locus**

One hundred SCCHN were examined for mutations in the INK4a/ARF locus utilizing a combination of PCR, “Cold” SSCP, and direct sequencing. Figure 6.1 shows a representative “Cold” SSCP analysis of a portion of exon 2 from three samples of tumor and matched normal tissues. Specimens 101 and 113 exhibit mutant SSCP bands in the tumor lanes (T), which migrate at different mobilities compared to their matched, normal control (N) and each other. A total of 27 samples (27%) exhibited sequence alterations in this locus. Somatic sequence alterations were identified in 22 of 100 (22%) tumor samples compared to the corresponding, patient-matched, normal tissue. An additional five samples (5%) exhibited a G to A transition at the first base of codon 148 of p16, in both the tumor and patient-matched normal samples, confirming previous reports of this population polymorphism (Hussussian, et al., 1994).

Table 6.1 summarizes the observed sequence changes in INK4a-ARF locus, and indicates the predicted consequences of the alterations on the p16 and p14ARF transcripts and proteins. Of the 22 SCCHN, in which somatic mutations were identified, twenty (91%) directly or indirectly involved alteration of exon 2, and two (9%) were located within exon 1α. No mutation was detected in exon 1β. All 22 mutations would be expected to yield an altered p16 protein sequence, but only 15 of them should affect the p14ARF protein. With reference to the mutations that affect p16 protein sequence, only 18% are missence mutations (samples #101, #156, #321, and #94-3581), and 27% are nonsense substitutions (samples #132, #133, #139, #93-2056, #94-916, and #95-1449).
Two samples (9%, samples #115, and #301) exhibited mutations within introns, which affected intron/exon splice junctions. Interestingly, three specific mutations within codons 54, 58, and 110 were identified in two different samples among those with nonsense substitutions. Several unique types of mutational events were identified in this large cohort of SCCHN. Of the 22 mutation-positive samples, a surprising 46% exhibited some type of small insertion or deletion. Among these unique mutations, three (14%) exhibited micro-deletions within the coding region of p16 ranging in size from 18 bp to 38 bp; one (5%) sample exhibited a micro-rearrangement consisting of the replacement of 37 bp of wild-type sequence with 128 bp of unknown sequence to yield a 91 bp net insertion; and six samples (27%) exhibited single or double nucleotide deletions or single nucleotide insertions. Recurrent tumor tissues were available from four patients [samples #63, #132, #139 #143], which provided us with an opportunity to independently confirm our initial results. In each instance, the recurrent tumors exhibited mutational events that were identical to those found in the corresponding primary tumor. Several of the mutational events have also been confirmed at the level of gene transcription where mRNA was available (Lang, et al., 1998).

With respect to the 15 mutations that affect p14AFR protein sequence, eight (53%) were missense mutations (samples #132, #133, #139, #321, #93-2056, #94-916, #94-3581, and #95-1449), six (40%) were small insertion or deletion (samples #61, #63, #143, #299, #93-3146, and #95-1495), and one sample (7%, sample #115) exhibited mutation within introns, which affected intron/exon splice junctions.
Figure 6.1  Representative examples of cold SSCP analysis of the \textit{INK4a/ARF} locus in SCCHN tumor (T), and patient-matched normal (N) tissues. Positive band shifts are apparent in the T lanes from sample 101 and 115 compared with the corresponding N lanes. Subsequent analysis of the PCR fragments by direct sequencing revealed mutation in both samples (Table 6.1).
Figure 6.1
<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Stage</th>
<th>Nucleotides change in locus</th>
<th>p16 Codon</th>
<th>p16 Coding</th>
<th>p16 Result</th>
<th>p14ARF Codon</th>
<th>p14ARF Coding</th>
<th>p14ARF Result</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>61</td>
<td>2</td>
<td>A → AA</td>
<td>54</td>
<td>1 bp insertion</td>
<td>FS</td>
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<td>FS</td>
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<td>76</td>
<td>R</td>
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<tr>
<td>143</td>
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<td>92</td>
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<tr>
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<td>-</td>
<td>-</td>
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<td><strong>Larynx</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>4</td>
<td>ATG...GTG</td>
<td>53-58</td>
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<td>T</td>
<td>67-73</td>
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<td>FS</td>
</tr>
<tr>
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<td>A → T</td>
<td>74</td>
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<td>M</td>
<td>88</td>
<td>No AA change</td>
<td>S</td>
</tr>
<tr>
<td>115</td>
<td>3</td>
<td>A → G</td>
<td>splice</td>
<td>Loss of exon 2</td>
<td>T</td>
<td>splice</td>
<td>Loss of exon 2</td>
<td>T</td>
</tr>
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<td>110</td>
<td>Trp → stop</td>
<td>T</td>
<td>125</td>
<td>Gly → A/g</td>
<td>M</td>
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<td>FS</td>
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<td>No AA change</td>
<td>-</td>
</tr>
<tr>
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<td>97</td>
<td>Leu → Pro</td>
<td>M</td>
<td>111</td>
<td>No AA change</td>
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<td>C,...,C</td>
<td>128-137</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>Ala → Thr</td>
<td>P</td>
<td>N/A</td>
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<td>-</td>
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<td>94</td>
<td>1 bp deletion</td>
<td>FS</td>
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<tr>
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<td>29</td>
<td>1 bp deletion</td>
<td>FS</td>
<td>N/A</td>
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<td>-</td>
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</table>

Table 6.1. Summary of INK4a/ARF locus alterations in SCCHN and their effect on p16 and p14ARF transcripts
Table 6.1 (continued)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stage</th>
<th>Nucleotide change in</th>
<th>Codon</th>
<th>Coding</th>
<th>Result</th>
<th>Codon</th>
<th>Coding</th>
<th>Result</th>
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<tbody>
<tr>
<td>Mouth</td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>132</td>
<td>4</td>
<td>C → T</td>
<td>58</td>
<td>Arg → stop</td>
<td>T</td>
<td>72</td>
<td>Pro → Leu</td>
<td>M</td>
</tr>
<tr>
<td>139</td>
<td>4</td>
<td>C → T</td>
<td>58</td>
<td>Arg → stop</td>
<td>T</td>
<td>72</td>
<td>Pro → Leu</td>
<td>M</td>
</tr>
<tr>
<td>301</td>
<td>4</td>
<td>T → A</td>
<td>splice Loss of exon 3</td>
<td>T</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>321</td>
<td>R</td>
<td>G → T</td>
<td>116</td>
<td>Glu → Tyr</td>
<td>M</td>
<td>130</td>
<td>Gly → Val</td>
<td>M</td>
</tr>
<tr>
<td>93-2056</td>
<td>na</td>
<td>G → T</td>
<td>88</td>
<td>Gln → stop</td>
<td>T</td>
<td>102</td>
<td>Gly → Val</td>
<td>M</td>
</tr>
<tr>
<td>93-3146</td>
<td>na</td>
<td>GGC..._G</td>
<td>56-68</td>
<td>38 bp deletion</td>
<td>T</td>
<td>70-82</td>
<td>38 bp deletion</td>
<td>FS</td>
</tr>
<tr>
<td>94-916</td>
<td>na</td>
<td>C → T</td>
<td>80</td>
<td>Arg → stop</td>
<td>T</td>
<td>94</td>
<td>Pro → Leu</td>
<td>M</td>
</tr>
<tr>
<td>94-2365</td>
<td>na</td>
<td>G → A</td>
<td>148</td>
<td>Ala → Thr</td>
<td>P</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>94-3581</td>
<td>na</td>
<td>C → G</td>
<td>63</td>
<td>Leu → Val</td>
<td>M</td>
<td>77</td>
<td>Ala → Gly</td>
<td>M</td>
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<td>94-3851</td>
<td>na</td>
<td>G → A</td>
<td>148</td>
<td>Ala → Thr</td>
<td>P</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>95-1449</td>
<td>na</td>
<td>G → A</td>
<td>110</td>
<td>Trp → stop</td>
<td>T</td>
<td>125</td>
<td>Gly → Arg</td>
<td>M</td>
</tr>
<tr>
<td>951495</td>
<td>na</td>
<td>A → AA</td>
<td>54</td>
<td>1 bp insertion</td>
<td>FS</td>
<td>68</td>
<td>1 bp insertion</td>
<td>FS</td>
</tr>
</tbody>
</table>

*R, recurrent; T, truncation; FS, frameshift; M, missense; S, silent; P, polymorphism; N/A, not applicable; na, not available.
The predicted protein coding changes induced by the 27 sequence changes were divided into four categories: i) truncation mutations (T) (40% in p16 and 7% in p14ARF), ii) frameshift mutations (FS) (26% in p16 and 36% in p14ARF), iii) missense mutations (M) (15% in p16 and 57% in p14ARF), and iv) polymorphisms (P) (19% only in p16).

**Site-directed Mutagenesis and Protein Purification of p16**

In an effort to determine the biological significance of the p16 mutations identified in this cohort of SCCHN, nine specific mutations were re-constructed using site-directed mutagenesis techniques, and functionally evaluated. The selected mutations represented three of the four mutation categories listed above: three truncation mutants, one frameshift mutant, and five missense mutants. No polymorphic mutants were evaluated since the codon 148 polymorphism was previously characterized by another group (Lilisschkis et al., 1996). All p16 recombinant genes were highly expressed in E. coli; however, five of the recombinant proteins had low solubility relative to wild-type p16 and most of the expressed proteins precipitated out as inclusion bodies in the host cells. The low availability of these recombinant p16 proteins in the soluble form necessitated their purification as GST fusion proteins (Figure 6.2A). These mutant proteins were also sensitive to thrombin digestion and were cleaved nonspecifically, making the removal of the GST domain unattainable (data not shown).

**Inhibition Activity of p16 mutants**

The functional integrity of the re-constructed mutant p16 was based on the ability to inhibit CDK4 kinase activity compared to wild-type P16. As a representative
truncation mutant, the GST-58stop mutant showed significantly lower inhibitory activity relative to wild-type GST-P16, a decrease of approximately 29-fold (Figure 6.2A). To the contrary, the GST-110stop mutant demonstrated only a 3-fold reduction in inhibitory activity. Interestingly, the GST-(53-58)Δ mutant, in which the turn connecting the two helices in ankyrin II (Figure 6.2B) of the protein is eliminated, only showed a 4-fold decrease in its inhibitory capacity. The GST-124fs frameshift mutant did not show apparent change in its inhibitory activity, suggesting that the C terminus is not crucial for the inhibitory activity of p16.

The missense mutants, which produce a single amino acid residue change, varied in their P16 inhibitory activity when ranked by the concentration necessary for 50% maximal inhibition (IC₅₀ values). But surprisingly, a dramatic residue change in GST-L97P did not result in a significant reduction in its inhibitory competence, suggesting that leucine 97 may not be crucial for the overall structure/function of p16. We were especially interested in codon 63 because it lies in helix IIB of p16, and is conserved in most ankyrin repeats. Therefore, we re-constructed not only the natural mutation observed in this study (L63V), but also two other missense mutations at this position, L63M and L63N. L63M, L63N and L63V had IC₅₀ values that increased 4- to 45-fold (Figure 6.2A), suggesting that even a conserved mutation at codon 63 could dramatically alter the inhibitory activity of p16. This further supports the finding that L63 has an important role in maintaining the global structure of p16. Detail scrutiny into the solution structure of p16 has provided us with a reasonable explanation to the above observation, which is addressed in the discussion section in this chapter.
Figure 6.2 (A) p16 mutant constructs and \textit{in vitro} CDK4 inhibition assay results. Schematic representation of the proteins and IC$_{50}$ values were obtained from the \textit{in vitro} CDK4 inhibition assay. (B) Topology diagram of the p16 structure. Helices (circles) are perpendicular to the plane of the page, and the residues forming helical secondary structure are indicated. * IC$_{50}$, concentration of the p16 protein that is required for 50% maximal CDK4 inhibition; N/D, non detectable; GST, glutathione S-transferase.
<table>
<thead>
<tr>
<th>GST (negative control)</th>
<th>GST (wild type; positive control)</th>
<th>Truncation</th>
<th>Amino Acid Substitution</th>
<th>Frameshift</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16 (wild type)</td>
<td>GST</td>
<td>GST-58stop</td>
<td>GST-L97P</td>
<td>GST-124fs</td>
</tr>
<tr>
<td>GST-p16 (wild type)</td>
<td>GST</td>
<td>GST-110stop</td>
<td>L63V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST</td>
<td>GST-(53-58)Δ</td>
<td>L63M</td>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>GST</td>
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<table>
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<th>IC₅₀ (10⁻⁹ M)</th>
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<tr>
<td>N/D</td>
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<td>630 ± 120</td>
</tr>
<tr>
<td>70 ± 20</td>
</tr>
</tbody>
</table>

Figure 6.2

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Figure 6.2 continued

B.
NMR spectroscopy of the p16 L63 mutants.

To study further the effect of mutagenic events in L63 on the global structure of p16, we employed one-dimension $^1$H NMR to check for possible structural perturbation. As demonstrated in Figure 6.3, all L63 mutants exhibited quite different spectra from that of wild-type p16. For L63N and L63V, most of the peaks at 6–9 ppm were greatly broadened, suggesting that the conformation of the mutants was significantly perturbed and/or the samples might have aggregated as a result of reduced protein solubility and stability. The spectrum of L63M was closer to that of wild-type P16, with only a modest broadening of the peaks, suggesting smaller perturbations in the structural properties. These findings are consistent with the corresponding in vitro CDK4 inhibitory activity of the mutants.
Figure 6.3  Comparison of one-dimensional NMR spectra among wild-type (WT), L63N, L63V, and L63M p16 proteins in $^2$H$_2$O at 600 MHz.
DISCUSSION

In the present study, we have assessed the importance of mutational inactivation of the INK4a-ARF locus in SCCHN, as well as the functional significance of several representative mutant p16 proteins identified in these tumors. Examination of 100 SCCHN revealed the presence of 27 (27%) genetic alterations within the locus. Five tumors exhibited the well-documented codon 148 polymorphism sequence change in p16 (Hussussian et al., 1994), as did the DNA from the corresponding patient-matched normal tissue. Twenty-two cases (22%) exhibited somatic mutations located predominantly within exon 2. Interestingly, recurrent tumors from four patients [samples 63.132.139.143] contained mutations identical to the patient-matched primary tumor. No mutation was observed in exon 1β, and only 15 of the 22 mutations would be expected to affect p14ARF proteins. The frequency of somatic p16 mutational events identified in this study is higher than that previously reported by others for SCCHN. In 15 SCCHN, Cairns et al. (1994) identified one somatic mutation (6%) in a more advanced tumor; Reed et al. (1996) found one mutation in 29 tumors (3%); Law et al. (1994) reported mutations in 6 of 48 (13%) HN tumors; Zhang et al. (1994) identified somatic mutations in 7 of 68 (10%) primary SCCHN; Uzawa et al. (1995) found somatic mutations in two of 32 (6%) oral cavity SCCs; and Kannan et al. (2000) identified in eight of 87 (9%) Indian oral SCCs. Taken together, these investigations found 25 somatic mutations out of 279 (9%) SCCHNs examined. The higher percentage of somatic mutation found in our study is statistically significant (P=0.01, chi-square test). Explanations for the increased frequency of p16 mutations observed in the present study...
may include differences in anatomical location and increased sensitivity of detection methodologies (Hongyo et al., 1993).

An interesting feature of this study is the prominent number (10 of 22, 45%) of micro-deletion and micro-insertion events identified in the SCCHN samples. All results were confirmed by duplicate experiments, and several of the mutations were confirmed in either recurrent tumor samples or primary tumor mRNA when available. A study by Yuedall et al. (1994) also demonstrated a high incidence (6/14, 43%) of small deletion/insertion mutations in cell lines derived from oral cancers. These unique mutations have also been reported in other tumor types, however at a lower incidence (Liu et al., 1995). One potential explanation for the high frequency of these micro-deletion/insertion events may be associated with a defective mismatch repair system within tumors. In eukaryotes, two heterodimers consisting of MSH2 and MSH6 or MSH2 and MSH3 have been shown to specifically recognize and repair DNA alterations in vitro and in vivo. MSH2's association with either MSH6 or MSH3 dictates the specificity of the type of mismatch. MSH6 targets base-base mismatches and small insertion/deletions for repair, whereas MSH3 recognizes only small insertions/deletions (Marra & Schar, 1999). Conceivably, dysfunction of one or more of the DNA repair proteins (e.g. MSH3) and/or disruption of the DNA repair pathways could lead to the accumulation of a specific type of mutational event within critical growth controlling genes such as p16 (Barnetson et al., 2000; Edelmann et al., 2000).

Another segment of this study was to determine the biological significance of the mutations identified within our cohort of SCCHN. There have been several site-directed mutagenesis studies done with p16 in the past several years, but most of the studies
focused on point mutations that result in a single amino acid residue substitution (Byeon et al., 1998; Li et al., 1999). The present study attempted to determine how more severe genetic and biologically relevant mutations, such as truncation and frameshift, affect the inhibitory activity of p16 on CDK4. We also evaluated four single amino acid substitution mutants that had not previously been examined in the in vitro inhibition activity assay. Lilischkis et al. (1996) examined a series of p16 deletion mutants and found that a truncation mutant, which comprises only the first and second ankyrin repeats, was void of inhibitory activity. In contrast, our results show that the truncated protein GST-58stop has an approximate 25-fold decrease in activity. This finding is consistent with an earlier report by Fahraeus et al. (1996) in which a chemically synthesized 20-amino acid peptide (corresponding to amino acid residues 39 to 57 of p16) had inhibitory activity for pRb phosphorylation. A possible explanation for the discrepancy between our results and those of Lilischkis et al. (1996) is that the IC_{50} value of the GST-58stop mutant is beyond the experimental upper limit established in the latter report. The GST-110stop and the GST-(53-58)Δ mutants had only a moderate decrease in activity, whereas the GST-124fs frameshift mutant retained full activity, similar to that of wild-type p16. These results are consistent with the findings of our previous biochemical and structural studies of p16, as well as the published CDK6/p16 complex crystal structure, wherein both the second and third ankyrin repeats of p16, but not the fourth ankyrin repeat, especially the C terminus are heavily involved in the interacting network with CDK6 (Byeon et al., 1998; Russo et al., 1998). However, it must be noted that the GST-124fs change may inflict other protein deregulating effects independent of the GST fusion protein. As discussed earlier, the GST-124fs is sensitive to thrombin.
digestion, and this could be very critical for the stability of this mutant protein in the cell. These results may explain why the level of mRNA expression in SCCHN primary tumors was highly elevated in our previous study (Lang et al., 1998), as the transformed cells may have increased gene expression in order to compensate for the reduced inhibitory activity of the mutant p16.

We also constructed five other missense mutants that had not been analyzed using the in vitro CDK inhibition activity assay. Leucine residues at positions 63 and 97 of the p16 protein are conserved among all INK4 family members, however GST-L97P and L63M mutants only exhibit 2-fold and 4-fold increases in IC50, respectively. On the other hand, the IC50 increases greatly for L63N and L63V. As revealed by the solution structure of p16 and the crystal structure of the CDK6/p16 complex, L63 does not directly interact with CDK6 (Byeon et al., 1998; Russo et al., 1998). Instead, it appears to play an important role in maintaining the structure of P16. As shown in Figure 6.4, L63 is located in the midst of a hydrophobic region, which stabilizes the helix bundle formed by the first and second ankyrin repeats of the protein. L63 forms hydrophobic interactions with L16, A17, A20, L32 (residues located on the first ankyrin repeat) and I49, V59, and V60 (residues located on the second ankyrin repeat). Any change in the side chain at this position may disrupt these interactions and destabilize the helix bundle in particular or the global structure of p16 as a whole. It is not difficult to comprehend the great increase in IC50 for L63N, however we find the remarkable difference in inhibitory ability/stability between L63M and L63V to be intriguing. The solution structure of p16 reveals that L63 is in close proximity with I49 and A48. Substitution of a
Figure 6.4  A partial structure of p16 that shows a hydrophobic interaction with L63 side chain. The structure was obtained from the restrained minimized mean NMR structure (PDB accession code 2A5E). L63 is a key hydrophobic residue which stabilizes the helix bundle formed by the first and second ankyrin repeats of p16.
leucine with a valine at this position results in the addition of two methyl groups at the C_{β} of the residue that may consequently inflict an undesirable steric effect within the hydrophobic core and therefore significantly perturb the global structure of p16. In addition, the length of the side chain of valine is shorter than that of leucine and may result in weaker hydrophobic interactions between residue 63 and its surrounding neighborhood. Since the side chain length of a methionine residue is comparable to that of leucine, and the C_{β} group of methionine is similar to leucine, the overall structure of the L63M mutant protein is more conserved and its inhibitory activity is better retained. Thus, the large increase in IC_{50} for L63V and L63N mutants is most likely the result of structural perturbation as suggested by the NMR spectra (Figure 6.3).

D116 is located on the first helix of the fourth ankyrin repeat of p16, and is not a conserved amino acid among INK4 proteins. Since the fourth ankyrin repeat is heavily involved in the interaction between p16 and CDK4/6, a mutation at this position would be unlikely to induce functional defect as detrimental as L63 mutation. Our in vitro inhibition assay confirmed this notion by showing that D110Y p16 mutant only elicited an eight-fold increase in IC_{50}, whereas L63V exhibited a thirty eighth-fold increase (data not shown).

One would expect a greater increase in the IC_{50} value for the L97P mutant, since a residue change to a proline at position 97 is likely to disrupt the helix bundle structure between ankyrin III and IV mediated by histidine 98. The greater degree of protein instability in this mutant is reflected in the fact that it is very insoluble in the host cells and is difficult to purify without the GST domain, which has a stabilizing role in protein expression and purification (data not shown). However, our previous biochemical studies
showed that mutations at V95, V96, and R99 did not cause significant changes in p16 structure and function (Li et al., 1999), suggesting that this region may be relatively flexible and a mutation at L97 may be tolerable. It should be noted that p16 is known to be very unstable and many of its mutants have been shown to present highly perturbed structures (Byeon et al., 1998; Li et al., 1999), therefore the in vitro structural and functional analysis of p16 and its mutants should only be interpreted qualitatively and with caution. Although more detailed structural and functional analyses of these mutants are highly warranted, such analyses are limited by the stability of these proteins.

In contrast to our structural and in vitro functional analyses of p16, similar evaluations of p14ARF mutants were impossible due to the nonavailability of the protein with high purity. However, it has been shown that the N-terminal 62 amino acids, as well as the 83-100 amino acid residues are important for the known functions of the protein, namely HDM2 binding, nucleolar localization, and the ability to induce p53-dependent cell cycle arrest. Mutations found in our study can be divided into two categories: (i) mutations that affect only p16 (samples #76, #101, #135, #156, #186, #191, #236, #249, #301, #302, #93-3146, and #94-2365), and (ii) mutations that affect both p16 and p14ARF (samples #61, #63, #115, #132, #133, #139, #143, #299, #321, #93-2056, #94-916, #95-1449, and #95-1495). To better understand the possible contribution of these two genes to the phenotypes observed in transformed cells, we were particularly interested in mutations found in samples #101, #156, #132, and #139. The mutation in sample 101 at this locus resulted in an amino acid residue change from aspartic acid to valine at codon 74 of p16 but silent in p14ARF. It has been reported that D74A has a perturbed structure, giving a defective protein (Zhang & Peng, 1996). Similarly, the mutation in sample 156
resulted in a residue change from leucine to proline at codon 97 in p16, but again silent in p14ARF. In vitro kinase inhibition assay of L97P mutant in our study only showed a two-fold increase in IC50 relative to wild-type. Although one would generally expect a greater increase in IC50 value for the proline mutant because a residue change to proline is likely to disrupt helix bundle structure, the greater degree of protein instability in this mutant may be reflected by the fact that it is very insoluble in the E. coli host cells, and is difficult to purify without the GST-domain (which has a stabilizing effect in protein expression and purification; data not shown).

Although detailed structural and functional analyses of p14ARF protein are highly warranted, they were deterred by the availability of this protein with high purity. The mutation in samples #132 and #139 (identical somatic mutation at this locus) resulted in a truncated mutant p16 protein but a missense alteration (P72L) in p14ARF. CDK4 inhibition assay showed a significant twenty five-fold increase in the IC50 value for the corresponding truncated p16 protein. However, codon 72 in p14ARF is not conserved between human and mouse. In addition, other studies have shown that amino acid substitution from a serine to an arginine and histidine residue in mouse p19ARF does not affect the growth-suppression potency of the protein in vivo (Herzog et al., 1999).

Overall, somatic mutational inactivation of the INK4a-ARF locus seems to be a significant event in the development of human SCCHN, an observation complements the well-documented INK4a-ARF-inactivation in various kinds of cancer. With the scores of evidence available to date, it is still difficult to pinpoint to which of the two gene products has a key role in cell transformation. Evidence that each may have a different function has been provided by studies in INK4a-ARF-null and ARF-null mice that have
different tumor spectra/phenotypes (Serrano et al., 1996; Kamijo et al., 1999). The contribution of these two gene products in tumorigenesis in specific tissue-types may be substantially dissimilar. Nonetheless, results from our studies indicate that p16 play a more important role than p14\textsuperscript{ARF} in SCCHN.
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