A BIOPHYSICAL STUDY OF HUMAN TYPE II INOSINE 5’ – MONOPHOSPHATE DEHYDROGENASE: IDENTIFICATION OF A REVERSIBLE SELF-ASSOCIATING SYSTEM AND AN ACTIVE MONOMERIC SPECIES.

by

Nicholas Peter Plaskos

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Biochemistry
In collaboration with the Institute of Biomaterials and Biomedical Engineering
University of Toronto

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ABSTRACT

Understanding how the structure of a protein affects its self-association characteristics and, perhaps more importantly, its interactions with other ligands are critically important for identifying its function in the body. We have undertaken a comprehensive investigation of human type II inosine 5'-monophosphate dehydrogenase (IMPDH-II), an enzyme involved in purine biosynthesis for replicating cells, by circular dichroism spectroscopy, mass spectrometry, non-denaturing PAGE, and in situ high resolution atomic force microscopy. While the structure of the tetrameric form of IMPDH-II has been reported, our studies have provided the first evidence that the enzyme exists in a reversible self-associating equilibrium state comprising monomeric, dimeric and tetrameric species, and that the monomeric form is functionally active. These studies establish a biophysical approach for investigating the structure-function-association characteristics of biomolecular complexes.
PREFACE

The work presented in this M.Sc. thesis was performed from 1998 – 2000 in the Canadian Center for Studies in Molecular Imaging (University of Toronto, Toronto, Ontario, M5S 3G9) under the co-supervision of Christopher M. Yip and Emil F. Pai. Financial support was provided by a University of Toronto Open Fellowship and an Ontario Student Opportunity Trust Fund. The results of this M.Sc. thesis are presented in two publications:

Plaskos N.P.; Pai, E.F.; Yip, C.M. In Situ Investigations of Lipid Assembly: Direct Visualization of Molecular Dissociation and Re-Incorporation During Bilayer Formation. 2000, In Preparation.

ACKNOWLEDGEMENTS

Never discourage anyone who continually makes progress, no matter how slow

-Plato

I am sure all graduate students will agree that this quote sums up perhaps the most important lesson learned from performing research. I would like to take this opportunity to thank all those that kept these words in mind and supported me along the way to my degree.

This work would not have been possible had it not been for remarkable supervision from Christopher Yip and Emil Pai. I would like to thank them for providing outstanding research environments and for continually inspiring me to strive for excellence. Chris' enthusiasm for knowledge and unparalleled work ethic gave me the opportunity to not only make the most out of my project, but also explore far beyond it. I am, however, still trying to figure out the "Yip Effect".

Of course I can not forget the Yip and Pai lab members and my fellow colleagues, who helped make grad life much easier and more enjoyable. I would like to thank Andrea Slade for spicing up lab life by never missing our daily, sometimes hourly, coffee runs and amazing everyone with her endless knowledge of jingles and songs. BFBH, just be free! Thanks also to Hemen Shukla, Elaine Lui, Hammy, and 'The Boys'.

I would also like to extend my sincerest appreciation to all those who contributed to my project. To Annie Cunningham and Steve Bryson, thanks for all your bench help, and to Joanne McLaurin and her lab group, your contributions have been invaluable. I would also like to thank Judi Martin for providing excellent proofreading services and actually making this thesis readable.

Finally, I can not express my gratitude enough to my family and Beth. You have all provided me with endless love and support and never let me give up on my dreams. This work is dedicated to you.
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Figure 4.4.11. (A) *In situ* tapping mode AFM height image acquired in pH 8 PBS buffer of discontinuous DMTAP bilayers after the addition of IMPDH-II to the imaging environment. (B) AFM phase image of (A) showing phase contrast between the existing DMTAP bilayer and the region of bilayer holes filled with IMPDH-II molecules. Image sizes: 2 μm x 2 μm. Scan rate: 1.97 Hz. Scale bar: 400 nm.

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Appendix 1B
Over-estimation of circular–shaped IMPDH-II molecules on DMPC bilayers

Appendix 1C
Over-estimation of circular–shaped IMPDH-II molecules on DMTAP bilayers
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAChe</td>
<td>Computer aided chemistry for education</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>6-Cl-IMP</td>
<td>6-chloropurine riboside 5'-monophosphate</td>
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<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-phosphatidylcholine</td>
</tr>
<tr>
<td>DMTAP</td>
<td>1,2-dimyristoyl-3-trimethylammonium-propane</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
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<tr>
<td>GRASP</td>
<td>Graphical representations of algorithms, structures, and processes</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>IMP</td>
<td>Inosine monophosphate</td>
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<td>IMPDH</td>
<td>Inosine monophosphate dehydrogenase</td>
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<td>IMPDH-I</td>
<td>Type I inosine monophosphate dehydrogenase</td>
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<tr>
<td>IMPDH-II</td>
<td>Type II inosine monophosphate dehydrogenase</td>
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<tr>
<td>LB</td>
<td>Langmuir-Blodgett</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MPA</td>
<td>Mycophenolic acid</td>
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<td>MS</td>
<td>Mass spectrometry</td>
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<td>Nicotinamide adenine dinucleotide</td>
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<td>Time of flight</td>
</tr>
<tr>
<td>XMP</td>
<td>Xanthine monophosphate</td>
</tr>
<tr>
<td>XMP*</td>
<td>Oxidized IMP thiomidate intermediate</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

1.1.1 Biosynthesis of Nucleotides

The biosynthesis of nucleotides is a vital biological process. Although many of the processes controlled by nucleotides are specific to their own metabolism, they also play key roles in many other biochemical processes.\(^1\) Perhaps most importantly, these compounds are essential precursors for the synthesis of both RNA and DNA. Without RNA production, protein synthesis cannot proceed, and unless other cells can manufacture DNA, they cannot divide. Nucleotides, such as cyclic AMP, act as metabolic regulators, while nucleotide derivatives are activated intermediates in many biosyntheses, such as glycogen and phosphoglyceride formation.\(^2\) Purine nucleotides regulate many important biochemical reactions associated with cell function.\(^1\) The adenine nucleotide ATP is a common source of energy in biological systems and a component of three major coenzymes: NAD\(^+\), FAD, and CoA.\(^2\) The guanine nucleotide GTP facilitates the movement of various macromolecules, such as the translocation of initial peptide chains on ribosomes and the activation of signal-coupling proteins.\(^2\)

Adenine and guanine nucleotides can be synthesized in three ways: from smaller precursors through the \textit{de novo} pathway, by recycling purine bases through the salvage pathway, or by phosphorylation of nucleosides.\(^2\) The \textit{de novo} and salvage pathways are the more important quantitatively,\(^2\) and it has been demonstrated that highly replicating cells rely heavily on the \textit{de novo} pathway. This is true for B and T lymphocytes, which
lack the salvage pathways and rely solely on the *de novo* pathway to provide purine nucleotides needed to fuel proliferative immune responses.³

1.1.2 The *De Novo* Pathway

The *de novo* pathway (Figure 1.1.1) plays an important role in cellular replication as it produces purine nucleotides needed to initiate the proliferative action by various cells.⁴ The first step of the *de novo* pathway is the phosphorylation of ribose 5'-phosphate using adenosine triphosphate (ATP) to form 5'-phosphoribosyl-1'-pyrophosphate (PRPP). PRPP is subsequently converted to 5-phosphoribosylamine, and then to inosine monophosphate (IMP). Here, IMP serves as the branch-point precursor for adenosine and guanosine nucleotides. Adenylosuccinate synthase converts IMP to adenylosuccinate, which is subsequently cleaved by adenylosuccinate lyase to form adenosine monophosphate (AMP). The enzyme inosine monophosphate dehydrogenase (IMPDH) oxidizes IMP to xanthine monophosphate (XMP) in the presence of nicotinamide adenine dinucleotide (NAD⁺).⁵ This is the committed step in guanosine nucleotide biosynthesis.⁶⁻⁸ In a series of reactions, XMP is serially converted to guanosine monophosphate (GMP), guanosine diphosphate (GDP), and finally phosphorylated to guanosine triphosphate (GTP), the most biologically active guanine nucleotide, via the action of the enzyme nucleoside diphosphokinase (NDPK).⁹ IMPDH, NDPK, and the rate of hydrolysis of GTP therefore control the cellular content of GTP. GTP is used for RNA, DNA, protein, and guanosine synthesis. Thus, IMPDH is a crucial determinant of the guanine nucleotides levels necessary for cell proliferation.
Figure 1.1.1. The de novo pathway of purine biosynthesis. Highly replicating cells rely primarily on this pathway as a source of purine nucleotides for DNA and RNA synthesis. IMP is the branch-point precursor for adenosine and guanosine nucleotide production. IMPDH regulates the committed step for guanosine nucleotide biosynthesis.
1.1.3 Inosine Monophosphate Dehydrogenase

1.1.3.1 General Overview

In 1957, purified extracts of Aerobacter aerogenes provided the first source of IMPDH. Since then, this enzyme has been isolated from various sources including mammalian, bacterial, parasitic, and plant. Recombinant expression systems have been constructed for human type I and II, Chinese hamster type II, Tritrichomonas foetus, Escherichia coli, Streptococcus pyogenes, Pneumocystis carinii, Borrelia burgdorferi, and Candida albicans IMPDHs. IMPDH is a dual isoform enzyme (Type I and Type II) and controls the rate-limiting step in de novo guanine nucleotide biosynthesis. There is a strong relationship between IMPDH activity and cellular proliferation and differentiation, thus illustrating the dependence of growing cells on de novo purine synthesis.

1.1.3.2 Global Structure

The crystal structure of IMPDH has been solved for a number of species, all but two of which contain a bound ligand such as a substrate, substrate analogue, product, or inhibitor. The Chinese hamster type II IMPDH structure was obtained at 2.6 Å resolution as a complex containing the covalently bound oxidized IMP thiomidate intermediate (XMP*) and the inhibitor mycophenolic acid (MPA). Since MPA binds in the nicotinamide binding region, this structure did not contain NAD+. Protozoan IMPDH from Tritrichomonas foetus was characterized in the apo form at 2.3 Å resolution and as the enzyme-XMP complex at 2.6 Å resolution. IMPDH from the pathogenic bacterium Streptococcus pyogenes was solved at 1.9 Å resolution with the substrate IMP present.
The structure of a ternary complex of human type II IMPDH with the halogenated substrate analogue 6-chloropurine riboside 5' - monophosphate (6-Cl-IMP) and the NAD\(^+\) analogue selenazole-4-carboxamide adenine dinucleotide (SAD) was recently reported,\(^{28}\) as was a 2.4 Å structure of substrate-free IMPDH from *Borrelia burgdorferi*.\(^{29}\) Together, the protozoan and bacterial forms share 30% - 40% sequence identities with known mammalian forms of IMPDH. The hamster form is highly homologous to the human form with only 6 different residues and has similar enzymatic and inhibitory characteristics with MPA.\(^{7}\) While there are significant differences in the active site environment among the solved forms of IMPDH, the general structure is conserved.

Native human IMPDH exists and is active as a ~ 222 kDa homotetramer with subunit monomer masses of ~ 55 kDa (Figure 1.1.2).\(^{5}\) IMPDH also crystallizes as a tetramer and this form is stabilized by contacts between adjacent monomers.\(^{7,28}\) In the

Figure 1.1.2. Native IMPDH-II exists as a tetramer.
hamster structure, specific inter-monomer contacts arise between residues near the N- and C-terminus of one subunit and amino acids part of or near the active site of an adjacent subunit. In the human form, specific protein-ligand contacts are observed between the adenosine portion of the dinucleotide analogue and each adjacent monomer. In either case, since all four active sites lie at the interface between subunits, these interactions may help to stabilize the active site conformation, particularly the cofactor binding region.

Composed of 514 residues, each monomer consists of two domains: a larger core domain that contains the active site and a smaller flanking domain (Figure 1.1.3). The ~400 residue core domain forms an eight-stranded parallel α/β barrel. The active site is located at the C-terminal end of the β-sheet barrel and is defined by specific loop and flap structures (residues 325-342 and 398-451 in the human form), along with interactions from an adjacent monomer (Figure 1.1.4). The active site comprises a long nucleotide

Figure 1.1.3. The IMPDH-II monomer consists of two domains: the core domain, which is a parallel α/β-barrel in which the active site is located, and the flanking domain.
binding cleft adjoining an NAD$^+$ binding groove.$^{28,29}$ The $\sim$ 100 residue flanking domain (residues 113-232 in the human form) protrudes from the N-terminal side of the barrel core adjacent to the catalytic domain, and lies between the second $\alpha$-helix and third $\beta$-sheet of the barrel.$^{28}$ The function of the flanking domain is not known, and apparently not required for activity as the truncated enzyme from $B. burgdorferi$$^{29}$ and Chinese hamster$^7$ retain full activity in vitro. In the hamster structure, the flanking domain is disordered and there is substantial size and sequence variability in this region among different species.$^7$ The angle between the core and flanking domain differs in the human and hamster forms, thus identifying a flexible hinge region at this junction.$^{28}$

Aside from the flanking domain, most solved IMPDH structures contain other disordered regions, including portions of the active site. The multiple conformations of the active site loop and flap imply some degree of conformational flexibility in IMPDH during the catalytic cycle. The loop and flap structures are disordered in the free and E-XMP complexed forms of the enzyme.$^{26,27}$ The loop contains the critical active site Cys-331 residue (Figure 1.1.4) which, along with portions of the flap, become ordered upon interaction with 6-Cl-IMP in the human structure.$^{28}$ Furthermore, the ability of Cys-331 to attack the C2, C6, and C8 substrate positions$^{26,30-32}$ demonstrates the flexibility of the loop region, which may be required for conformational transitions between the various enzyme-ligand states during the course of a normal reaction. The flap structure also exhibits some degree of flexibility as regions of the flap are disordered prior to cofactor binding.$^{7,26,28,29}$ A conformational change occurs when the adenosine portion of NAD$^+$ interacts with certain sections of the flap thus ordering it. The flap structure in the human type II isoform was based on fitting a region of continuous well-ordered density with a
10-residue turn and 5-residue section of β-sheet.\textsuperscript{28} Although side chain density was observed here, explicit residue assignments could not be made nor could the fragments be unambiguously traced back to the main chain.\textsuperscript{28} Thus, the residues were not included in the final model and the flap structure (white) depicted in Figure 1.1.4 is based on a previously hypothesized schematic model.\textsuperscript{28}

Figure 1.1.4. Specific loop (green) and flap (white) structures, along with interactions from the adjacent monomer (red) define the active site region of IMPDH-II. Binding of the substrate analogue, 6-Cl-IMP (light blue), induces a localized conformational change by causing the loop structure to adopt a more compact arrangement as it collapses around the nucleotide. This ordering of the loop also facilitates the interaction of Cys-331 (yellow) with 6-Cl-IMP. Binding of the NAD\textsuperscript+ analogue, SAD (pink), causes sections of the active site flap to make contact with the adenosine portion of the dinucleotide and stabilize its binding.
1.1.3.3 Mechanism of Action

In the presence of NAD\(^+\), IMPDH catalyzes the oxidation of IMP to XMP, with the subsequent production of reduced nicotinamide adenine dinucleotide (NADH).\(^{11} \) In the absence of substrate and/or cofactor, the IMPDH active site loop and flap segments are disordered, resulting in an open conformation around the nucleotide binding site.\(^{12,26,28,29,34,35} \)

IMP binds in an *anti* conformation for the glycosidic bond and a C2-*endo*-conformation for the sugar ring (Figure 1.1.5).\(^{36} \) This organization of the inosine base keeps C2 away from the sugar ring and facilitates its interaction with Cys-331.\(^{36,37} \)

![Diagram illustrating 6-Cl-IMP and SAD binding.](image)

Figure 1.1.5. Diagram illustrating 6-Cl-IMP and SAD binding. 6-Cl-IMP binds in the *anti*-conformation for the glycosidic bond and a C2-*endo*-conformation for the sugar ring. The dinucleotide ring of SAD stacks in the *anti*-position against the 6-Cl-IMP purine ring.

Nucleophilic attack by the active site sulfur at Cys-331 on C2 of IMP results in a covalent thio-linkage.\(^{28} \) Binding of IMP induces a localized conformational change by causing
the loop to adopt a more compact and stable arrangement as it collapses around the nucleotide. All known structures of IMPDH contain this loop structure between β6 and α6 of the barrel, and the IMP-induced conformational change appears to be a general feature of the IMPDH reaction mechanism. In both the Chinese hamster and human type II forms the loop covers the bound substrate, however, the loop in the human-complexed structure adopts a more extended conformation in order to accommodate the substrate analogue 6-Cl-IMP. Close inspection of the hamster and human enzyme structures revealed that the phosphate binding site is completed by closing of this loop, which contains a highly conserved serine involved in interactions with the phosphate moiety of IMP. Upon binding of the substrate, the active site flap collapses slightly on the nicotinamide end of the cofactor pocket. This partially stabilized conformation, caused by collapse of the loop and partial closure of the flap, has a higher affinity for NAD⁺.²⁸

NAD⁺ binds in an extended conformation similar to that observed in other dehydrogenases with the nicotinamide moiety stacking against the base of IMP. This orientation was also noted of the NAD⁺ analogue, SAD, in the human structure (Figure 1.1.5).²⁸ This orientation is important since the nicotinamide ring must be oriented to allow hydride transfer from the C2 position of IMP to the C4 position of NAD⁺. This results in the glycosidic portions of both the nicotinamide-ribose and adenosine moieties binding in the anti conformation.²⁸ Hydride transfer would occur at the B-side of the nicotinamide ring consistent with the fact that IMPDH is a B-side specific enzyme.²⁹ Once a hydride is transferred to NAD⁺, a covalently bound oxidized XMP* and NADH in a ternary complex with the enzyme is obtained. The XMP* intermediate is hydrolyzed to XMP in a subsequent step. Binding of NAD⁺ causes the putative sections of the active
site flap to make contact with the adenosine portion of the dinucleotide (Figure 1.1.4). These contacts viewed in the human type II form would continue the series of interactions between IMP and the flap witnessed in the hamster complex, and suggest that the active site flap serves primarily to stabilize the dinucleotide. Stabilization upon binding of NAD$^+$ was confirmed as closure of the flap and loop affords enhanced protection of the substrate and cofactor against proteolysis.

Finally, there is an ordered release of products with XMP following NADH. A free catalytic water molecule hydrolyzes XMP* to form XMP. It has been proposed that this catalytic water interacts with Thr-333 and Gln-441 and would be properly oriented for nucleophilic attack at C2 of the XMP* intermediate. However, this hypothesis is currently being tested. This portion of the IMPDH reaction mechanism is not well understood. Nevertheless, the active site remains in the 'closed' configuration throughout the catalytic steps and reverts to the 'open' conformation with XMP release via retraction of the active site loop and flap.

1.1.3.4 KINETIC PROPERTIES OF IMPDH

1.1.3.4.1 Activity

Kinetic studies determined that optimal activity for human IMPDH occurs at an approximate pH of 8. IMPDH from various species can be denatured with 3M - 8M urea and renatured with retention of activity. The IMPDH-catalyzed reaction follows simple Michaelis-Menten kinetics, which suggests that the affinity of all four active sites is approximately equal. Recently, however, Bruzzese and Connelly detected negative cooperativity within the four IMP binding sites in human IMPDH using
titration – calorimetry experiments. While the first three IMP molecules bind with similar affinity, the affinity for the last IMP molecule is decreased approximately 10-fold.

Early experiments revealed a sequential reaction mechanism, wherein both substrates bind to the enzyme before either product is released. The data were also fit to the steady state ordered Bi-Bi velocity equation, which further indicated that IMP binding precedes that of NAD+, and NADH is released prior to XMP. Thus, it has been argued that IMPDH follows an ordered Bi-Bi reaction sequence where the substrates bind and the products are released in an obligate order. This mechanism is different from that of most other known NAD+ dependent dehydrogenases, which have either a random order of substrate addition or require that NAD+ bind before the substrate. These conclusions, however, were based on product inhibition experiments that are only valid if no intermediate form(s) are present. Since the intermediate, E~XMP*, is known to exist in the reaction scheme of IMPDH, it has been suggested that IMPDH follows a different reaction mechanism based on a random addition of substrate and cofactor, and an ordered release of products with NADH preceding XMP.

It has been found that IMPDH from various organisms require a monovalent cation, such as K+, Na+, Rb+, NH4+, or Tl+, in order to elicit maximum activity. Thus, the steady state ordered Bi-Bi reaction mechanism Carr et al. used to describe the IMPDH-catalyzed reaction was not complete since it did not include the monovalent cation. The activation of human IMPDH-II by various monovalent cations has been characterized and an approximate 100-fold activation by K+ was found. Kinetic experiments with human IMPDH revealed that although IMP can bind to the enzyme in the absence of potassium, activity was less than 1% of that in the presence of K+. These
results revealed the requirement of $K^+$ as an essential activator for human IMPDH activity and thus the need to include it in the steady state reaction mechanism. Thus, in the complete reaction sequence, IMPDH binds the monovalent cation first and then IMP and NAD$^+$ are bound in a random order (Figure 1.1.6). NADH is then released before XMP. As with most other $K^+$-stimulated enzymes, however, the mechanistic basis for the rate enhancement by the monovalent cation is unknown.

Figure 1.1.6. The complete reaction sequence of IMPDH. The monovalent cation is bound first, then IMP and NAD$^+$ are bound in a random order, and finally XMP is released following NADH.

1.1.3.4.2 Inhibition

IMPDH is subject to substrate inhibition by high concentrations of NAD$^+$ as this is common when there is an ordered release of products. NAD$^+$ inhibition results from the formation of an E-XMP*-NAD$^+$ complex$^{19}$ and is uncompetitive with respect to IMP.$^{41}$ The values of $K_i$ for NAD$^+$ range from 0.6-3 mM among IMPDHs from different sources.$^{35,45,48}$ It is important to note that intracellular NAD$^+$ concentrations are $\geq$ 400 $\mu$M. This indicates that a significant population of IMPDH exists in vivo as the E-XMP*-NAD$^+$ complex. In addition, the $K_{cat}$ values for reactions with various NAD$^+$ analogs are equivalent suggesting that the rate-limiting step for human IMPDH must be a step that does not involve NAD$^+$ or NADH. Furthermore, kinetic isotope studies showed that hydride transfer is fast, reversible, and not rate-limiting as no $V_{max}$ isotope effects are
observed when 2-²H-IMP is the substrate. These studies also demonstrated that hydrolysis of E-XMP* and/or XMP release is rate-limiting in the IMPDH reaction.

1.1.3.4.3 Self-Association – Dissociation Characteristics

It is very important to note that the self-association and –dissociation properties of human IMPDH have not been thoroughly investigated. Glycerol density gradient centrifugation found that both isoforms of human IMPDH sedimented as native tetramers. An early study indicated that IMPDH from Aerobacter aerogenes consisted of various isomers ranging from 90,000 to 950,000 Da. This study utilized ultracentrifugation and sedimentation analysis to show that the enzyme undergoes dissociation as ionic strength is increased and demonstrated reversible loss of activity after urea denaturation. The fundamental catalytic species was believed to have a molecular weight of 90,000 – 100,000 Da and possess one IMP per molecule. It was thus identified as the monomeric form. A subsequent study on IMPDH also from Aerobacter aerogenes confirmed the presence of a mixture of molecular weight isomers, all of which retained some degree of activity. Furthermore the isomers demonstrated dissociation with increasing ionic strength, urea, and reducing agent concentrations. Under conditions comparable to those used for kinetic analysis, it was determined that the enzyme had a molecular weight of ~ 86,000 Da and this was the smallest active species observed. The amino acid composition and peptide map revealed that this species was made up of two identical polypeptide chains and thus was a dimer. A more recent study of IMPDH from Tritrichomonas foetus reported tetramer dimerization into 444 kDa octomers with an equilibrium dissociation constant of 1 – 2 µM. Thus, although native IMPDH exists and
is active as a tetramer, it appears that under certain conditions this enzyme may demonstrate reversible self-associating characteristic with the possibility that other multimeric species retain activity.

1.1.3.5 HUMAN TYPE I AND TYPE II IMPDH ISOFORMS

Isoforms of human IMPDH, designated as Type I (IMPDH-I) and Type II (IMPDH-II), have been identified. Containing 514 amino acids, the IMPDH-I and IMPDH-II isoforms show 84% sequence identity. They exhibit similar affinities for substrate and cofactor, IMP and NAD+, and follow similar enzymatic mechanisms. The isoforms also have similar $K_{cat}$ values of 1.5 and 1.3 turnovers/molecule/second of enzyme at 37°C for Types I and II. In the IMP site, the only residue that is not conserved between the human isoforms is residue 327. In Type I this residue is Cys-327 and in Type II it is Ser-327. There is considerably more difference in the NAD+ site between the isoforms. The greatest difference is the number of nonconserved residues at the terminal end of the flap region. Despite the many similarities, the isoforms appear to have different roles. The Type I isoform is constitutive and dominant in most cell lines. The Type II isoform is the predominant form found in highly replicating cells, particularly B and T cells, which are critical in providing the proliferative responses needed in the immune response to foreign agents. This response system can cause problems, however, such as rejecting a transplanted organ or mistaking an organism's normal cells as foreign. Several studies have shown that immunosuppression via inhibiting IMPDH-II is effective in the treatment of refractory rejection in renal, heart, and liver transplant recipients, and may be effective in the treatment of chronic
As IMPDH-II plays a major role in cellular proliferation, it is selectively up-regulated and predominant in highly replicating and neoplastic cells. Increased IMPDH-II activity also has been observed in rapidly proliferating human leukemic cell lines, solid tumor tissues, lymphocytic and acute myeloid leukemias. As uncontrolled cellular proliferation is the hallmark of cancerous tissue, it is common in the treatment of cancer to use compounds that specifically inhibit this process. There are significant therapeutic advantages to be gained by selectively inhibiting IMPDH-II for immunosuppressive and cancer therapies.

1.1.4 IMPDH-II Inhibitors

Major IMPDH-II inhibitors can be classified into two groups based upon the mode of enzyme binding. One group of inhibitors are IMP analogues that are phosphorylated intracellularly to the corresponding 5'-monophosphates, which then binds to the substrate (IMP) site. The active metabolites of mizoribine and ribavirin are the IMP analogs MMP and RMP, respectively (Figure 1.1.7). A second group of inhibitors are NAD⁺ analogues and bind to the NAD⁺ site. The active metabolites of tiazofurin and selenazofurin are the NAD⁺ analogs TAD and SAD, respectively (Figure 1.1.7). Mycophenolic acid (MPA), although not a nucleoside, also binds in the NAD⁺ site of IMPDH.
1.1.4.1 MYCOPHENOLIC ACID

1.1.4.1.1 General Overview

Discovered in 1898 as a fermentation product of several *Penicillin* species,\textsuperscript{5,7,65} MPA was initially studied for possible antibiotic and antiviral use and later displayed considerable anti-tumour activity.\textsuperscript{65} The general mechanism of action of MPA is based on interference with purine synthesis by selective inhibition of IMPDH-II.\textsuperscript{67} Of the NAD\textsuperscript{+} binding site inhibitors, MPA binds with the highest affinity and specificity for IMPDH-II.\textsuperscript{67} Since all the residues identified as interacting with MPA are conserved between the two isoforms,\textsuperscript{7} this specificity may be due to subtle conformational differences in the MPA binding site. As mentioned, highly proliferating cells rely primarily on the *de novo*
pathway for purine synthesis and use IMPDH-II, the predominant isoform in these cells, as opposed to nonreplicating cell lines that rely on the IMPDH-I isoform. In the de novo purine synthesis pathway, inhibition of IMPDH-II by MPA creates a mechanism for depleting guanosine nucleotides, which results in selective inhibition of highly replicating cells, including T and B lymphocytes, with minimal effects on other organ systems. This is most likely due to the approximately 5-fold higher binding affinity MPA has for IMPDH-II over IMPDH-I. Despite this selectivity between isoforms, however, the role of MPA as an antitumour agent has not been thoroughly investigated.

1.1.4.1.2 Mechanism of Action

MPA is a heterocyclic molecule (Figure 1.1.8) and a potent inhibitor of mammalian IMPDHs but a poor inhibitor of microbial IMPDHs. MPA acts as an NAD⁺ analogue and is an uncompetitive inhibitor with respect to both IMP and NAD⁺, indicating that both substrates bind to the enzymes before MPA binds.

![Figure 1.1.8. MPA is a heterocyclic molecule and a potent inhibitor of IMPDH-II.](attachment:image.png)
Data from Carr et al.\textsuperscript{5} were consistent with MPA binding to either the E-IMP-NAD\textsuperscript{+}/E~XMP*-NADH ternary complexes or the E-XMP/E~XMP* complexes. It was later discovered that MPA binds exclusively to the E~XMP* complex.\textsuperscript{24,68} MPA binds to the nicotinamide site for NAD\textsuperscript{+} after NADH is released but before XMP is produced (Figure 1.1.9),\textsuperscript{69} thus preventing full conversion of IMP to XMP by trapping the covalently bound XMP* intermediate before the final hydrolysis step.\textsuperscript{34} The structure of the E~XMP*-MPA complex revealed that MPA is packed against the purine base of IMP, analogous to how the nicotinamide portion of NAD\textsuperscript{+} would.\textsuperscript{7} The MPA bicyclic ring forms H-bonds with residues forming the pocket walls.\textsuperscript{7} The MPA hexenoic ‘tail’ forms hydrogen bonds with residues from the stabilizing flap and floor of the groove.\textsuperscript{7} Comparison of X-ray structures of MPA and NAD\textsuperscript{+} revealed that based on comparable molecular volumes and electrostatic characteristics, the lactone, carbonyl, carboxylate, and the aromatic substituents of MPA could be superimposed on the carboxamide, phosphate, and pyridine moieties of NAD\textsuperscript{+}.\textsuperscript{69} The structure of the IMPDH-II/MPA complex also indicates that the hydroxyl substituent of MPA may substitute for the catalytic water, which is hydrolyzed to produce XMP.\textsuperscript{7} These considerations suggest that MPA may act as both an NAD\textsuperscript{+} and catalytic water mimic.

\begin{center}
\begin{tikzpicture}

\node (K) at (0,0) {$K^+$};
\node (IMP) [right of=K] {$\text{IMP}$};
\node (E-IMP) [right of=IMP] {$E-\text{IMP}$};
\node (E-XMP*) [right of=E-IMP] {$E-\text{XMP}^*$};
\node (E-XMP) [right of=E-XMP*] {$E-\text{XMP}$};
\node (XMP) [right of=E-XMP] {$\text{XMP}$};
\node (MPA) [below of=E-XMP] {$\text{MPA}$};
\node (H2O) [above of=E-XMP] {$H_2O$};
\node (NAD+) [below of=IMP] {$\text{NAD}^+$};
\node (NAD) [below of=NAD+] {$\text{NAD}^+$};
\node (NADH) [below of=NAD] {$\text{NADH}$};

\draw[->] (K) -- (IMP);
\draw[->] (IMP) -- (E-IMP);
\draw[->] (E-IMP) -- (E-XMP*);
\draw[->] (E-XMP*) -- (E-XMP);
\draw[->] (E-XMP) -- (XMP);
\draw[->] (XMP) -- (MPA);
\draw[->] (MPA) -- (H2O);
\draw[->] (H2O) -- (E-XMP);
\draw[->] (E-XMP) -- (NADH);
\draw[->] (NADH) -- (NAD);
\draw[->] (NAD) -- (NAD+);
\draw[->] (NAD+) -- (IMP);

\end{tikzpicture}
\end{center}

\textit{Figure 1.1.9.} MPA acts as an NAD\textsuperscript{+} analogue and binds to the E~XMP* complex after NADH is released but before XMP is produced. ‘E’ denotes free IMPDH-II.
1.1.4.1.3 Kinetics of Inhibition

MPA binds specifically to the E-XMP* complexes in human type II IMPDH. The affinity of MPA for E-IMP is almost $10^3$-fold lower and no binding to free enzyme was observed. This inhibitor also has an approximate 5-fold higher binding affinity for IMPDH-II over IMPDH-I. MPA displayed inhibition constants on the order of $10^{-8} - 10^{-9}$ M against IMPDH, which classifies it as a tight binding inhibitor. When enzyme and inhibitor concentrations are equivalent, tight binding inhibitors deviate from standard steady state assumptions. Under these conditions, this effect must be taken into account in determining the mechanism of inhibition and the calculation of $K_i$ values. Using the standard steady state analysis, the $K_i$ at 5 nM enzyme for MPA against type I IMPDH was 37 nM, and that against type II was 9.5 nM, producing a 3.9-fold difference in $K_i$ between the two isoforms. Using the tight binding treatment, the $K_i$ values were 33 nM for type I and 7 nM for type II, producing a 4.8-fold difference. Since MPA binds to E-XMP*, the value of $K_i$ will also depend on the accumulation of E-XMP*.

1.1.5 Effects of IMPDH-II Inhibition

Many aspects of cellular metabolism require an adequate supply of guanine nucleotides and reduction of this nucleotide pool by IMPDH-II inhibition has been shown to result in a variety of therapeutically useful effects. IMPDH-II inhibitors have been exploited as antiviral, immunosuppressive, and antileukemic agents. Inhibition of IMPDH-II affects nucleic acid synthesis, gene expression, signaling and, ultimately, cell proliferation and differentiation. The inhibition of IMPDH-II and subsequent reduction in guanine nucleotide levels interrupts DNA and RNA synthesis in rapidly
dividing tumour cells.\textsuperscript{73} Furthermore, there is down-regulation of c-myc and/or Ki-ras oncogenes in a variety of human tumour cell lines\textsuperscript{70,78,79} and in blast cells of leukemic patients treated with the inhibitor.\textsuperscript{70} The inhibition of IMPDH-II also appears to compromise the ability of G proteins to function as transducers of intracellular signals.\textsuperscript{72,74-77} IMPDH-II inhibitors have been successfully employed in immunosuppressive therapy as they inhibit mitogen-induced proliferation of human T and B lymphocytes.\textsuperscript{67}

\section*{1.1.6 Atomic Force Microscopy}

\subsection*{1.1.6.1 General Overview}

Atomic Force Microscopy (AFM), a member of the scanning probe microscopy (SPM) family, provides an effective complement to spectroscopic and diffraction-based approaches for studying the structure of biomolecules. This technique has provided new insights into the structure of interfaces and surfaces,\textsuperscript{80} including correlation of local surface topographies with specific sample properties at the near-molecular scale.\textsuperscript{81} The ability to perform such measurements in liquids and in real-time has led to the application of AFM to other areas, including studies of enzyme structures,\textsuperscript{81,83} characterization of drug-DNA complexes,\textsuperscript{84} and the \textit{in situ} measurement of attractive forces between molecules.\textsuperscript{85,86} This high-resolution sensing technique has provided excellent insights into the structure of inorganic,\textsuperscript{87} molecular,\textsuperscript{88,89} and protein crystals.\textsuperscript{87,90}

AFM is also well suited for providing detailed topographical information about biomolecular structures adsorbed at the solid-liquid interface. Not only is this technique suitable for examining the structure of \textit{individual} molecules,\textsuperscript{91-97} but the ability to
perform such measurements *in situ* and in real-time suggests the possibility of observing dynamic biomolecular *complex* formation as it occurs under physiological conditions.\(^{98-100}\)

1.1.6.2 **AFM Operational Information**

An AFM system typically comprises a piezoelectric scanner, a microfabricated imaging tip, an optical beam, and a detection system. The silicon nitride AFM tip is mounted into a holder positioned over the sample previously fixed to a piezoelectric scanner. The scanner physically rasteres the sample in a line-by-line fashion in the x-y plane during imaging, and the position of the tip over each (x,y) position on the sample provides the z-directed motion used to map surface topography.\(^{101}\) The AFM detection system includes a computer controller that monitors and modifies the operation of the scanner *via* a feedback mechanism. The feedback signal is generated by a laser beam deflection system (Figure 1.1.10). A laser diode generates a spot of light that is reflected off the back of the AFM cantilever onto a mirror and finally onto a position sensitive photodetector. As the tip raster scans over the sample surface, features on the surface deflect the cantilever, which changes the position of the laser spot on the photodiode. The displacement in the position of the reflected laser spot relative to a setpoint position is used to generate the error signal to the feedback control mechanism. This feedback control moves the tip by adjusting the z-piezo position to return the reflected laser spot to the null setpoint position on the photodiode. The motion in the z direction required to return the tip to its null position provides a measure of the surface topography.
There are two major operating modes of AFM – contact mode and TappingMode™ (TMAFM). In contact mode AFM, the above process is done while the tip images at a constant force on the sample (Figure 1.1.11A). A problem with this mode of operation, however, is the frictional force that exerts torque on the cantilever, which may disturb the sample.\textsuperscript{101} TMAFM involves vertically oscillating the tip at high frequency and constant amplitude and scans the surface, using intermittent contact (Figure 1.1.11B). The amplitude of tip oscillation serves as the signal for the AFM detection system. As the microscope scans a sample, height variations on the surface of
the sample will cause the oscillating amplitude of the tip to change. The computer controller detects this change in amplitude as an error signal and returns a feedback control signal to the microscope to regain the original tip oscillation amplitude. The tip oscillation is kept constant by moving the sample in the z direction as the tip is simultaneously translated in the x, y direction. This is transformed into a topographical image by the computer, which is displayed in real-time. A key advantage to this technique, which is especially important for soft materials, is the substantial reduction in lateral shear forces present during contact AFM imaging.

Figure 1.1.11. Tip movement in (A) contact and (B) TappingMode™ mode AFM.

One of the advantages of AFM lies in the ability to simultaneously collect complementary information while scanning. In the case of TMAFM, this information is commonly height, which measures vertical height of the sample surface, amplitude, which represents the magnitude of the error signal, and phase, which measures differences in surface properties such as viscoelasticity, adhesion, and electrostatics.102,103
In phase imaging the detected phase lag of the cantilever oscillation relative to the signal sent to the cantilever's piezo driver is simultaneously monitored by the feedback controller.

TMAFM, while far less destructive to the surface, yields slightly reduced lateral resolution due to the intermittent contact with the sample surface. Furthermore, the parameters used to control the imaging process are particularly important to image quality. In particular, the cantilever drive amplitude and setpoint voltages, which together determine the cantilever oscillation magnitude to be controlled by the feedback circuit during imaging. These parameters vary from sample to sample and are optimized with respect to image resolution and contrast, and sample integrity.

1.1.6.3 LIMITATIONS OF AFM

The most relevant issues regarding limitations of AFM revolve around the interactions between the tip and sample. The scanning motion of the tip can disrupt the surface structure resulting in tip – induced damage. TMAFM in fluid can cause 'growth' artifacts due to a stirring motion during scanning. Another important consideration is wearing of the tip or accumulation of debris on the end of the tip.\textsuperscript{104} The finite tip dimensions cause both of these events to occur, and this often results in distortion of the image. As the imaging mechanism of these scanning probe techniques is based on the interaction of the tip with a substrate, the information which is acquired is highly location specific and, thus, an appropriate number of samples must be taken in order to reach a general conclusion about surface structure and mechanics. The resolution capabilities of AFM also remain a controversial issue. The AFM imaging mechanism relies on the
interaction forces between the tip and sample that cannot be easily measured directly and are indirectly determined by deflection of a cantilever spring. Thus, it is important to note that interpretation of high-resolution scanning probe data requires correlation against known structural data as well as careful consideration of image artifacts.

1.1.7 Supported Lipid Bilayers

Recent studies have demonstrated that supported lipid bilayers (SPBs) are effective membrane mimics. These structures have proven ideal for investigating fundamental bilayer properties and characteristics including domain structure, physical stability, defect formation, and blistering. Supported lipid bilayers have found uses as biosensors, templates for protein crystallization, and as substrates for studies of antibiotic action, ion channel activity, and the forces associated with ligand binding events. Lipid bilayer structure has been extensively characterized by spectroscopy, Brewster angle microscopy, fluorescence, near-field optical microscopies, and recently scanning probe techniques. To date, many of these initiatives have focused on identifying lipid domain structures, phase segregation phenomena, and molecular diffusion processes on samples prepared by Langmuir-Blodgett (LB) techniques. Under these conditions, wherein the lipid solution of interest is dispersed at the air-water interface and subsequently transferred under surface pressure to a solid substrate, the resulting lipid films are typically highly ordered. While appropriate for preparing mono- and multi-layers with well-defined structural characteristics, this approach is inappropriate for investigating the initial stages of lipid assembly at solid interfaces. An alternative approach for preparing bilayer (and multilayer) structures is
simply the direct adsorption of small unilamellar lipid vesicles from solution onto the solid support.\textsuperscript{120} Since vesicle fusion allows bilayer formation to occur without the imposition of external forces, the resulting structures are in a highly fluid state and thus provide an appropriate membrane-mimetic substrate for studying lipid diffusion, membrane assembly processes, protein-lipid and protein-protein interactions. In fact, SPBs are ideal substrates for immobilizing proteins and applying surface-dependant techniques such as AFM. They provide a natural environment that is molecularly smooth, biocompatible, and the existence of a wide range of phospholipids allows flexibility in terms of tailoring surface charge. This is particularly important in order to examine differences in orientations or, more significantly, to direct specific orientations in order for the possibility of subsequent real-time imaging of substrate-enzyme interactions.

1.2 HYPOTHESIS

In the past, the structure of free and complexed human IMPDH-II has been studied by traditional methods including various spectroscopies and diffraction-based approaches. Conventional biochemical techniques have also been used to characterize features such as kinetics and physical properties of the isoforms, acid/base-dependent catalysis, and the thermodynamics associated with ligand binding. It should be noted that most of these techniques used sample concentrations that are much greater than those required for a conventional enzyme activity assay. Taking this into consideration, a few fundamental questions arise.
Although the native tetrameric structure of human IMPDH-II has been resolved for many years, is the tetramer the only possible form or can other mono or multimeric IMPDH-II structures exist? Evidence of different molecular weight isomers has been detected in IMPDH from *Aerobacter aerogenes*.\(^{30,42}\) Furthermore, this is a possibility since the concentration of IMPDH-II used in structural studies, such as X-ray crystallography, is much higher compared to those used in an activity assay or even other biochemical techniques. At higher protein concentrations, it would be expected to see the prevalence of the tetrameric or even higher multimeric forms according to the principles governing reversible self-associating systems.\(^{121}\) Following the same principles, however, at very dilute concentrations an equilibrium between monomers, dimers, trimers, and tetramers should exist.

Another question that arises is that although the tetrameric structure has been determined to be the active species, does the monomeric enzyme retain any catalytic activity? Each monomer contains an active site and structural studies have indicated that the active site is located near the interface of adjacent subunits within the tetramer. Inter-monomer contacts have been identified where residues of one subunit core domain make contact with residues that are part of the active site of an adjacent subunit. These contacts stabilize the tetrameric structure of the enzyme and the cofactor binding portion of the active site conformation. From this, it has been postulated that these contacts are required for activity of the enzyme. While these contacts may help stabilize a portion of the active site to a certain extent, the specific role that the contacts play in relation to their effect on monomeric activity have not been defined. The entire activity of each monomer may not hinge upon the presence of these inter-monomer contacts and thus, even in their
absence, the IMPDH monomer may retain some degree of activity. Similar results were demonstrated for IMPDH from *Aerobacter aerogenes*, as the smallest species isolated was capable of activity.\(^{30,42}\)

Therefore, the hypothesis of this thesis was two-fold. Firstly, although the native structure of IMPDH-II has been characterized as a tetramer, at dilute concentrations there would be the existence of mono and other multimeric enzyme populations. Secondly, the monomeric species of IMPDH-II would retain some degree of activity, even in the absence of stabilizing inter-monomer contacts.

### 1.3 OUTLINE

A biophysical study of IMPDH-II was performed at concentrations near those used in a conventional enzyme activity assay in order to determine the size populations present, and whether the monomeric species retained any activity. This study began with a biochemical characterization by the traditional techniques of circular dichroism spectroscopy, mass spectrometry, and non-denaturing polyacrylamide gel electrophoresis. Crystallographic studies on Chinese hamster IMPDH-II indicated that adjacent monomers participate in creating secondary structural elements in the tetrameric structure. Thus, dissociation of the tetramer may be accompanied by disruption of these secondary structural elements. As the participating residues are fully conserved in human IMPDH-II, this dissociation-based structural change could also occur in this form and may be detected and estimated by circular dichroism spectroscopy. This technique has displayed the requisite sensitivity to measure similar secondary structure changes on the same order of magnitude.\(^{122}\) The presence of different molecular weight species within a
homogenous protein sample can also be detected by mass spectrometry.\(^{123}\) Non-denaturing polyacrylamide gel electrophoresis is suitable for separating different sized species within a protein sample based on size, shape, and charge. This method also allows for the utilization of the separated protein bands by other techniques. In this case, protein bands corresponding to IMPDH-II monomers and multimers were transferred to a membrane via a western blot transfer,\(^{124}\) and then used in a conventional activity assay to determine if they possessed any activity. The application of the relatively novel technique, atomic force microscopy, was then used to elucidate the structure(s) of IMPDH-II molecules at concentrations corresponding to those used in the biochemical characterizations. Supported planar bilayers were used as biomimetic substrates for the analysis of human IMPDH-II structure adsorbed to lipid interfaces.

The combination of these powerful biochemical techniques along with a high-resolution structural elucidation tool has created an effective biophysical characterization study of IMPDH-II that clearly addressed the key concerns of this thesis.
1.4 REFERENCES


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CHAPTER 2

Biochemical Characterization of Human type II Inosine 5′ - Monophosphate Dehydrogenase

2.1 OVERVIEW

In humans, proliferating cells are dependent on the de novo pathway for purine biosynthesis. The committed step for guanine nucleotide biosynthesis is controlled by the enzyme inosine 5′-monophosphate dehydrogenase (IMPDH). IMPDH exists in two isoforms, type I (IMPDH-I) and type II (IMPDH-II), and the latter is found at up-regulated levels in highly replicating and neoplastic cells. Native IMPDH-II has been characterized as a tetramer and it has been postulated that this form is required for activity. Since diffraction-based techniques use relatively concentrated samples, we performed a biochemical characterization of this enzyme at concentrations near those used in a conventional enzyme assay in order to determine the structures present at these low concentrations. Circular dichroism (CD) analysis revealed that a 100-fold reduction in IMPDH concentration to 0.02 mg/ml resulted in a significant 7 % reduction in β-sheet content. These results are consistent with dissociation of the IMPDH tetramer since its formation results in an increase in β-sheet content due to the creation of parallel and anti-parallel β-sheets at the interface between adjacent IMPDH monomers. This model is further supported by mass spectrometry (MS) and non-denaturing polyacrylamide gel electrophoresis (PAGE) analyses, which revealed the existence of a dynamic equilibrium between IMPDH-II monomers, dimers, and tetramers. Furthermore, activity assays using monomeric IMPDH-II, separated by non-denaturing PAGE and electroblotted to a
polyvinylidene difluoride (PVDF) membrane, demonstrated that the monomeric form retains activity.
2.2 MATERIALS

Purified IMPDH-II at 20 mg/ml in dialysis buffer (50 mM TRIS-HCL/8.0, 300 mM KCl, 10% Glycerol, 2 mM βMe, 2 mM EDTA) was provided by Mr. Steve Bryson and Mrs. Annie Cunningham (Biochemistry, University of Toronto) and stored at -70 °C in 50 μl aliquots. All electrophoresis reagents and pre-cast gels were purchased from BioRad (Mississauga, ON, CAN). PVDF membrane was purchased from Biotechnology Systems (Boston, MA, USA). Evaluation of circular dichroism spectra was performed using the CDsstr computer program. Water was distilled and deionized using Millipore Milli-Q UF Plus (Bedford, MA, USA) at 18 MΩ resistance. All other reagents were of the highest analytical grade.
2.3 METHODS

2.3.1 Circular Dichroism Spectroscopy

IMPDH-II was diluted to 2 mg/ml, 0.10 mg/ml, and 0.02 mg/ml using pH 8 PBS buffer (150 mM NaCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄ unless otherwise specified). Quartz cuvettes were thoroughly cleaned using 10 M NaOH and rinsed with deionized water and sample CD spectrums were recorded on a Jasco Circular Dichroism Spectrometer Model J-715 (Easton, MD, USA) at 25°C. Spectra were recorded from 195-250 nm, with a 0.5 nm step size, 1 nm bandwidth, and 1 second collection time per step. Spectra were averaged from 5 repeat scans and smoothed using Jasco system software. After subtracting the buffer control the data were converted to mean-residue molar ellipticities using a monomer molecular weight of 55,500 and a number of amino acids of 514. The CD spectra were analyzed using the program CDsstr, which uses variable selection combinations of known protein CD spectra in a basis set to predict the secondary structure content of the proteins. The program predicts the secondary structure content of a protein for: α-helix, 3₁₀-helix, poly(L-proline) II type 3₁-helix, β-sheet, β-turn, and other structures. Our results included all α-helical structures into one category termed “Total α-helix”. It has been found that the presence of high levels of NaCl may interfere with CD analysis, thus all CD experiments were repeated using PBS buffer with 50 mM NaCl and no differences were observed (data not shown).
2.3.2 Mass Spectrometry

The University of Toronto Mass Spectrometry Laboratory performed mass spectrometry experiments. IMPDH-II was diluted to 2.0 mg/ml and 0.10 mg/ml using pH 8 PBS (50 mM NaCl, 10 mM Na2HPO4, 10 mM NaH2PO4). Protein samples were analyzed with positive ionization mode on a Voyager-DE STR matrix assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometer (Perseptive Biosystems Inc., Farmingham, MA, USA), equipped with a pulsed nitrogen laser (337 nm, 3-ns pulse). 1 µl of protein solution was spotted on a MALDI plate and 1 µl of matrix (saturated sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) dissolved in aqueous 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA)) was applied over the protein solution. The MALDI plate was inserted into the instrument after the solution on the plate was air-dried at room temperature. Operating parameters include: accelerating voltage 25 KV, grid voltage 95.00% of accelerating voltage, guide wire voltage 0.150% of accelerating voltage, delay time 320 ns. Bovine serum albumin (BSA) was used for external calibration.

2.3.3 Non-Denaturing Polyacrylamide Gel Electrophoresis

IMPDH-II was diluted to 2.0, 0.10, and 0.02 mg/ml using pH 8.0 PBS buffer. Each sample, along with NOVEX SeeBlue™ Pre-stained Standards, was then mixed 1:1 with native sample buffer (1.5 M TRIS, pH 8.8, 20% glycerol, 0.1% bromophenol blue). Using a 50 µl Hamilton glass syringe, ~ 40 µl of each of the 4 samples were loaded into lanes 1 to 4 and this was repeated in lanes 6 to 9 of a 4-12% pre-cast gradient gel. The gel was inserted into a NOVEX EI9001–XCELL II™ Mini Cell apparatus (Helixx
Technologies, Ontario, CAN). The gel was subjected to a 4 hr, 100 mV bias after which the gel was removed and divided into two equal sections by lane number. Lanes 1 through 4 were developed using a NOVEX SilverXpress Silver Staining Kit (Helixx Technologies, Ontario, CAN), photographed, and dried overnight in drying solution (20% methanol, 2% glycerol) using a NOVEX gel drying apparatus. The other half of the gel containing lanes 6 to 9 was immediately used in a Western blot protein transfer experiment.

2.3.4 Western Blot Protein Transfer

Western blot protein transfer experiments were performed on lanes 6 through 9 according to standard protocols. The transfer was effected onto PVDF membranes using a NOVEL XCELL II Blot Module (Helixx Technologies, Ontario, CAN). The membrane was then developed using PONCOUE stain and thoroughly washed prior to sealing and freezing at −70 °C.

2.3.5 Protein Dot Blot

The PVDF membrane was soaked in methanol for approximately 10 minutes and transferred into a Dot Blot apparatus. 10 μg, 1 μg, and 0.60 μg IMPDH-II, diluted with pH 8 PBS to 0.2 mg/ml, 0.02 mg/ml, and 0.012 mg/ml respectively, were added to the apparatus in 50 μl dot quantities and transferred to the membrane via vacuum suction. The membrane was then immediately sealed and frozen at −70 °C.
2.3.6 IMPDH-II Activity Assays

As a control, a conventional enzyme activity assay was performed on IMPDH-II (see below). This was compared to activity assays of IMPDH-II adsorbed to PVDF membrane from dot blot and Western transfer experiments. Portions of PVDF membranes that contained whole protein from the protein dot blot were excised and stored in pH 8.0 TRIS. PVDF membranes used in the protein transfers were unwrapped and thawed, and bands were excised from the 0.02 mg/ml IMPDH-II lane and stored in pH 8.0 TRIS buffer. The first half of the gel containing lanes 1 to 4, which was now dried, was used as a template to determine the approximate location of the protein bands to be excised on PVDF membranes. The activity assay solution contained 50 mM TRIS, pH 8.0, 100mM KCl, 3mM EDTA, 1mM NAD, 1mM IMP, and 10 μg of IMPDH-II (for the control experiment). IMPDH-II activity was determined spectrophotometrically by monitoring the increase in solution absorbance at 340 nm, which is indicative of the formation of NADH, with an UltraSpec 3000 spectrophotometer (Pharmacia Biotech; Piscataway, NJ, USA) at 5, 10, 15, 30, 45, and 60 minutes. Reactions were initiated by the addition of the soluble enzyme (for the control) or excised portions of enzyme-bound PVDF membrane. The assay solution and PVDF membrane band mixtures were constantly agitated within the cuvette (using an automatic stir rod) in between spectrophotometric measurements and the PVDF membrane remained in the cuvette during absorption readings. Measurements were performed at 37°C and set with an upper absorbance limit of 0.1. Negative control absorption measurements of PVDF membranes were taken at similar time intervals. Control activity experiments were also performed in order to determine if IMPDH-II remained bound to the PVDF membrane.
2.4 RESULTS

2.4.1 Circular Dichroism Spectroscopy

CD spectra were obtained for IMPDH-II at concentrations of 2.0, 0.10, and 0.02 mg/ml in pH 8 PBS. As the concentration decreased, the CD spectra changed from a single large minimum at ~ 224 nm to two minima at ~ 209 nm and 223 nm (Figure 2.4.1). The relative measurements of secondary structure for the 2.0 mg/ml samples were 15 % Total α-helix, 32 % β-sheet, and 14 % β-turn. For the 0.10 mg/ml samples: 29 % Total α-helix, 28 % β-sheet, and 9 % β-turn; and for 0.02 mg/ml samples: 30 % Total α-helix, 25 % β-sheet, and 11 % β-turn. Secondary structure measurements were based on five sets of experimental results. Refer to Table 2.4.1 for a summary of the results. It should be noted here that the CDssstr program gives a root mean square error of 3.3% for α-helix, 2.6% for 3_{10}-helix, 2.7% for poly(L-proline) II type 3_{1}-helix, 4.2% for β-strand, 4.2% for β-turn, and 5.1% for other structures.
Figure 2.4.1. CD spectroscopy spectra were obtained for IMPDH-II at 2.0, 0.10, and 0.02 mg/ml concentrations. As the concentration decreased, the spectra were characterized by a shift from a single large minimum at approximately 224 nm to two minima at approximately 209 nm and 223 nm.

<table>
<thead>
<tr>
<th>Conc. Of IMPDH-II (mg/ml)</th>
<th>Total α-Helix</th>
<th>β-Sheet</th>
<th>β-Turn</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>15 ± 2</td>
<td>32 ± 3</td>
<td>14 ± 1</td>
<td>39 ± 1</td>
<td>100</td>
</tr>
<tr>
<td>0.10</td>
<td>29 ± 1</td>
<td>28 ± 1</td>
<td>9 ± 2</td>
<td>34 ± 1</td>
<td>100</td>
</tr>
<tr>
<td>0.02</td>
<td>30 ± 0</td>
<td>25 ± 1</td>
<td>11 ± 1</td>
<td>34 ± 2</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.4.1. Secondary structure analysis on circular dichroism spectra for IMPDH-II at 2 mg/ml, 0.10 mg/ml, and 0.02 mg/ml revealing relative percentages of Total α-helix, β-sheet, β-turn and other structures based on estimations performed by CDsstr protein secondary structure program. Each value is a mean ± S.D. of five determinations.
2.4.2 Mass Spectrometry

Mass spectrometry results revealed the existence of several different mass populations in each of the 2.0 mg/ml and 0.10 mg/ml IMDPH-II samples. In the 2.0 mg/ml sample, there were five primary intensity peaks at average mass ratios (m/z) of 55,751.3, 83,290.3, 111,032.1, 165,732.1, and 220,797.0 (Figure 2.4.2). The average relative abundances at each of these values were 261, 39, 75, 33, and 19 respectively. In the 0.10 mg/ml sample, there were four primary intensity peaks at average mass ratios (m/z) of 55,901.2, 84,278.5, 111,952.9, and 166,485.9, with an additional intensity peak noted at 223,833.0 (Figure 2.4.3). The average relative abundances at each of these values were 651, 31, 67, 20, and 14 respectively. Size populations and relative abundances were based on five sets of experimental results. Refer to Table 2.4.2 for a summary of the results.

![Mass spectrometry spectrum of IMPDH-II at 2.0 mg/ml showing the relative abundance at each major size population.](image)

Figure 2.4.2. Mass spectrometry spectrum of IMPDH-II at 2.0 mg/ml showing the relative abundance at each major size population.
Figure 2.4.3. Mass spectrometry spectrum of IMPDH-II at 0.10 mg/ml showing the relative abundance at each major size population.

<table>
<thead>
<tr>
<th>Conc. of IMPDH -II (mg/ml)</th>
<th>Relative Abundance of Various Size Populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td><img src="#" alt="Table 2.4.2" /></td>
</tr>
<tr>
<td>0.10</td>
<td><img src="#" alt="Table 2.4.2" /></td>
</tr>
</tbody>
</table>

Table 2.4.2. Mass spectrometry of IMPDH-II at 2.0 mg/ml and 0.10 mg/ml revealing the average relative abundance for major size populations at each concentration. The average m/z values are indicated in parentheses. Each value is a mean ± S.D. of five determinations.
2.4.3 Non-Denaturing Polyacrylamide Gel Electrophoresis

Silver-stained non-denaturing PAGE of IMPDH-II at 2.0, 0.10, and 0.02 mg/ml revealed bands at approximately 55 kDa, 110 kDa, and 220 kDa (Figure 2.4.4). The 110 and 220 kDa bands became more defined as the sample concentration decreased. The 55 kDa band was very clear and intense at 0.02 mg/ml and was not evident in the other concentrations.

Figure 2.4.4. Silver-stained non-denaturing PAGE of IMPDH-II at (1) 2.0, (2) 0.10, and (3) 0.02 mg/ml concentrations. Staining revealed bands at approximately 55 kDa, 110 kDa, and 220 kDa. In each lane 0.60 µg of protein was loaded.
### 2.4.4 IMPDH-II Activity Assays

#### 2.4.4.1 Control Activity Assay

<table>
<thead>
<tr>
<th>Mass of IMPDH Used (µg)</th>
<th>Absorbance at 340 nm at specific time intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>10</td>
<td>0.040</td>
</tr>
<tr>
<td>1</td>
<td>0.009</td>
</tr>
<tr>
<td>0.60</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 2.4.3. Absorbance readings at 340 nm at specific time intervals measuring the activity of IMPDH-II at 10, 1, and 0.60 µg quantities in a conventional enzyme activity assay. Each value is the mean of five determinations.
2.4.4.2 IMPDH-II Activity Assays from PVDF Membranes

Negative control experiments on PVDF membranes showed no activity.

2.4.4.2.1 Dot Blot IMPDH-II

<table>
<thead>
<tr>
<th>Mass of IMPDH used (μg)</th>
<th>Absorbance at 340 nm at specific time intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>10</td>
<td>0.033 ± 0.008</td>
</tr>
<tr>
<td>1</td>
<td>0.007 ± 0.006</td>
</tr>
<tr>
<td>0.60</td>
<td>0.000 ± 0.000</td>
</tr>
</tbody>
</table>

Table 2.4.4. Absorbance readings at 340 nm measuring the activity of 10, 1, and 0.60 μg IMPDH-II quantities that had been transferred to a PVDF membrane by dot blotting and used in an enzyme activity assay. Each value is a mean ± S.D. of five determinations.
2.4.4.2.2 Western Transfer IMPDH-II

Negative control experiments on PVDF membranes showed no activity. Control experiments also revealed that IMPDH-II remained bound to the PVDF membranes during the course of the activity assay experiments.

<table>
<thead>
<tr>
<th>IMPDH -II 0.60 µg</th>
<th>Absorbance at 340 nm at specific time intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Tetramer</td>
<td>0.001 ± 0.000</td>
</tr>
<tr>
<td>Dimer</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>Monomer</td>
<td>0.021 ± 0.006</td>
</tr>
<tr>
<td>Total</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Table 2.4.5. Absorbance readings at 340 nm measuring the activity of 0.60 µg IMPDH-II separated into tetramer, dimer, and monomer bands and transferred to a PVDF membrane for use in an enzyme activity assay. Each value is a mean ± S.D. of five determinations.
2.5 DISCUSSION

2.5.1 Circular Dichroism Spectroscopy

CD spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of left- and right-handed circularly polarized light by a substance. The absorption of circularly polarized light by individual chromophores occurs to different degrees and at different wavelengths. A chromophore is a functional group with identifiable electronic transitions that are loosely coupled with the rest of the molecule. For proteins, the most abundant chromophore is the peptide group. The long range order or disorder of the peptide chromophore within a folded protein is responsible for the characteristic spectra of each of the secondary structural elements. CD spectroscopy is a highly sensitive and convenient method for following folding and unfolding transitions in globular proteins as it is very sensitive to the secondary structure of proteins and polypeptides. Far–UV spectra can be analyzed for the different secondary structural types: \( \alpha \)-helix, parallel and anti-parallel \( \beta \)-sheet, \( \beta \)-turn, and random coil, while near–UV spectra provides details regarding tertiary structure.

The \( \alpha \)-helix is the dominant secondary structure in many proteins and on the average accounts for about 1/3 of the residues in globular proteins. The spectroscopic characterization of the \( \alpha \)-helix has been greatly facilitated since its conformation is well defined. The CD spectra of an \( \alpha \)-helical protein are negative at 222 nm, due to the peptide \( n-\pi^* \) transition, and negative at 208 nm and positive at 192, due to exciton splitting of the peptide \( \pi-\pi^* \) transition. The characterization of the \( \beta \)-sheet is more difficult than for the \( \alpha \)-helix since the \( \beta \) conformation is less well defined structurally. The pleated sheet can vary in both length and width, while the helix varies only in length.
The CD spectra of a β-sheet are negative at 218 nm, due to a $n\rightarrow\pi^*$ transition, and positive at 195 nm, due to a $\pi\rightarrow\pi^*$ transition. Lastly, in globular proteins, there are always regions of the polypeptide chain that are unordered or random. This is characterized by a strong negative band just below 200 nm due to the $n\rightarrow\pi^*$ transition, and varies in the positive region above 200 nm, due to the $\pi\rightarrow\pi^*$ transition. CD spectroscopy is an effective technique to characterize the secondary structure of proteins in solution. Modifications of secondary structure induced by changes of protein concentration, pH, and salt associated with self-associating equilibriums were detected by far-UV CD spectra. Highly analogous to IMPDH-II, a study examining poly(ethyleneglycol)-insulin conjugates revealed that dimerization of insulin monomers involves the creation of an antiparallel β-sheet at the subunit interface and was detected by shifts in CD spectra.

We have applied far-UV CD spectrometry to study the secondary structure of IMPDH-II. Visual inspection of the CD spectra of IMPDH-II as the concentration decreased from 2.0 to 0.02 mg/ml revealed a shift from one large negative minimum at ~224 nm to two distinct minima at ~209 and ~223. At 2.0 mg/ml, the minimum at 224 nm is at a higher wavelength than the expected value of 218 nm for a β-sheet, however, the highly variable nature of this structure allows for some degree of flexibility in the wavelength. The organization of the β-sheet can be in the parallel or anti-parallel manner and, in keeping with this variability of the β-sheet conformation, the CD spectra of this structure shows pronounced deviations. Large spectral variations from 218 nm have been reported for β-sheet structures, with minima occurring as high as 225 nm for species with high β-sheet content. At 2.0 mg/ml, the β-sheet and α-helical content for IMPDH-II were calculated to be approximately 32% and 15% respectively. At 0.02 mg/ml, the
two minima at 209 nm and 223 nm are very close to the typical 208 nm and 222 nm that represent α-helical character. At this concentration, the β-sheet and α-helical content were calculated to be approximately 25% and 30% respectively.

Sintchak et al.² solved the crystal structure of Chinese hamster IMPDH-II, and noted the creation of secondary structural elements at various points of contact between adjacent subunits (Figure 2.5.1A). Residues 41 – 43 form a parallel β-sheet with residues 279 – 281 in an adjacent subunit (depicted in black). Also, residues 507 – 510 form an anti-parallel β-sheet with residues 444 – 447 of the active site flap of an adjacent subunit (depicted in grey). The hamster and human forms of IMPDH-II are highly homologous, differing in only 6 residues. The residues participating in the formation of the parallel β-sheet (residues 41, 43, 279, 281) are completely conserved in the human form, and when we examine the structure of the human tetramer it is clear that a similar parallel β-sheet is also formed at the interface between these residues (Figure 2.5.1B; depicted in black). It is likely that the formation of the anti-parallel β-sheet also exists in the human form, however, these residues (444, 447, 507, 510) were not resolved in the human IMPDH-II structure presented by Colby et al.¹

The formation of these β-sheet structures at the monomer interfaces of the IMPDH-II tetramer may be related to the spectral shift observed with a change in protein concentration. CD analysis revealed a loss in β-sheet content as the concentration decreases from 2.0 mg/ml to 0.02 mg/ml. As this concentration shift occurs, there may be a concurrent shift in self-association equilibrium to favour the formation of monomers. This dissociation would lead to the disruption of the intermolecularly generated parallel and anti-parallel β-sheets.
Figure 2.5.1. β-sheet formation at the interface of IMPDH-II monomers in the tetrameric structure. In the hamster form (A), parallel and anti-parallel β-sheets are formed by residues 41-43 and 279-281 (shown in black), and residues 444-447 and 507-510 (shown in grey) of adjacent monomers. In the human form (B), a similar parallel β-sheet is formed at residues 41-43 and 279-281, however, residues involved with the anti-parallel β-sheet are not reported in the electron density map.
It should be noted here that we observed greater β-sheet content for 2.0 mg/ml IMPDH-II than that found by Nimmesgern et al.\textsuperscript{11} In this study, pH 8 PBS buffer (150 mM NaCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 10 mM NaH\textsubscript{2}PO\textsubscript{4}) was used while Nimmesgern et al. used CD buffer containing 100mM pH 8 potassium phosphate buffer, 10% glycerol, 2 mM β-mercaptoethanol. The reducing agent β-mercaptoethanol hinders aggregation by preventing disulfide bond formation between free cysteine residues on the surface of IMPDH-II monomers. Glycerol also helps to prevent aggregation by increasing the hydration state of a protein. It is therefore reasonable to suggest that, in the absence of glycerol and a reducing agent, aggregation may occur at 2.0 mg/ml. It has been shown that there is a very close relationship between β-sheet formation and aggregation within proteins.\textsuperscript{12-16} There would be greater β-sheet content at this concentration and this would explain the strong and slightly shifted β-sheet signal at 224 nm. Further, at 0.02 mg/ml, where aggregation would not likely be taking place, we observed less β-sheet than Nimmesgern et al. This supports our contention that at low concentrations, there is loss of β-sheet content due to dissociation of IMPDH-II tetramers.

This concentration-dependent study of IMPDH-II secondary structure by CD revealed that as protein concentration decreased, there was a gradual loss of β-sheet content. Further, at 0.02 mg/ml IMPDH-II, the CD spectra revealed that the amount of β-sheet detected was lower than that previously found to exist at 2 mg/ml. This loss in β-sheet content may be due to a self-association equilibrium shift towards monomers with decreasing protein concentration, which is accompanied by the disruption of parallel and anti-parallel β-sheets at subunit interfaces of the tetramer.
2.5.2 Mass Spectrometry

The matrix assisted laser desorption ionization (MALDI) technique is used for measurement of the molecular masses of proteins and other large biomolecules that are thermally unstable. Indeed, this becomes the major application for this soft or ‘mild’ method of ionization since it minimizes sample fragmentation. This technique is highly precise, with an accuracy of about 0.1%. In some cases it is possible to acquire useful mass spectral data for proteins with molecular masses over 200,000 Da. Furthermore, when analyzing large peptides and proteins, this technique produces an uncluttered background spectrum with ions due to the matrix occurring at m/z values below 1,000. TOF analyzers, which are commonly used with MALDI, separate ions of different masses by making use of their different velocities after acceleration through an electric field. The velocity of an ion is dependent on mass, thus a collection of ions can be readily separated based on their velocities and thus arrival times at the detector. This method of analysis affords this technique fast response time.

To acquire a MALDI-TOF spectrum, a small amount of the sample is added to a large excess of matrix molecules in solution, typically sinapinic acid dissolved in aqueous 50% ACN and 0.1% TFA, and allowed to air dry on a metal target. In this case, the sample (IMPDH-II) was mixed with a solid organic matrix – sinapinic acid dissolved in aqueous 50% ACN and 0.1% TFA. The target is loaded into the ion source and irradiated with short intense pulses from the laser. The matrix solution strongly absorbs the wavelength(s) emitted by the laser (337 nm) and controls the energy subsequently deposited in the sample molecules which are desorbed as protonated molecules, [M + H]^+. In a typical laser desorption mass spectrum of a protein, both cluster ions, [nM +
w+, and multiply charged ions, [M + zH]^z+, may be detected. It is critical to consider the solution used to prepare the samples. Very low levels of some salts, buffers, and detergents, as well as less than 2% of glycerol can be tolerated.

It has been determined by X-ray crystallography that IMPDH-II exists as a tetramer in solution with subunit masses of approximately 55,500 kDa.\(^1\) In this study, 2.0 and 0.10 mg/ml IMPDH-II in pH 8 PBS (50 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4) were analyzed by mass spectroscopy in order to determine the size of the species present in solution at these concentrations. The PBS buffers used throughout this thesis contain 150 mM NaCl; however, a lower concentration (50 mM) was used for mass spectroscopy since higher levels of salt interfere with the technique. At 2.0 mg/ml, there were 4 noticeable size populations, with an additional small peak slightly above the baseline. At average m/z values of 55,751.3, 83,290.3, 111,032.1, and 165,732.1, the relative abundances were 261, 39, 75, and 33 respectively. A minor peak was found at an average m/z value of 220,797.0 with a relative abundance of 19. Eliminating the mass added by the charged species, this results in relative masses of 55,750.3, 83,289.3, 111,031.1, 165,731.1, and 220,796.0 Da respectively.

At 0.10 mg/ml, there were 3 noticeable size populations, with an additional small peak slightly above the baseline. At average m/z values of 55,901.2, 84,278.5, and 111,952.9, the relative abundances were 651, 31, and 67. The minor peak was at an average m/z value of 166,485.9 with a relative abundance of 20. If we examine the portion of the spectrum corresponding to the 220,797.0 m/z peak in the 2.0 mg/ml sample, we also note a small peak at 223,833.0 with a relative abundance of 14 in the 0.10 mg/ml spectra. Eliminating the mass added by the charged species, this results in
relative masses of 55,900.2, 84,277.5, 111,951.9, 166,484.9, and 223,832.0 Da respectively.

In each sample, the values of the relative masses as determined by mass spectrometry most likely represent the masses of actual protein species in solution. A common artifact of this technique, however, is the presence of clusters (multiples) of the analyte, which depends on the structure of the sample and its concentration in the matrix. Generally, the greater the concentration of the sample, the greater the extent of the clustering (i.e. [2M + H]^+, [3M + H]^+, etc.). Since native IMPDH-II has been determined to exist as a ~ 222,000 Da tetramer, clusters would be present at multiples of this size.

At a concentration of 2.0 mg/ml, there was a very large peak at 55,750.3, a peak at 111,031.1, and 3 smaller peaks at 83,289.3, 165,731.1, and 220,796.0. At a concentration of 0.10 mg/ml, there was a large peak observed at 55,900.2, a peak at 111,951.9, and 2 smaller peaks at 84,277.5 and 168,371.8. An additional peak was noted at 223,832.0 through a comparison with this region of the spectra at 2.0 mg/ml. At concentrations below 0.10 mg/ml IMPDH-II, there was insufficient signal to discern sample mass peaks from the baseline. Thus, at 0.10 mg/ml the possibility of clustering would be very low due to the dilute concentration of the sample. Both concentrations show no mass above 220,000 Da and thus clustering of tetrameric IMPDH-II is not taking place.

The masses detected in each sample correspond to the actual masses of the IMPDH-II monomer (~ 55,500 Da), dimer (~ 111,000 Da), trimer (~ 166,500 Da), and tetramer (~ 222,000 Da). The ~ 84,000 Da mass identified by MALDI-TOF may be an additional IMPDH-II structure. The IMPDH-II flanking domain is a highly disordered
structure and it is connected to the core domain be a series of residues described as a flexible hinge region.\textsuperscript{2} The expected molecular weight of the IMPDH-II flanking domain is \( \sim 16,700 \text{ Da} \),\textsuperscript{11} thus the \( \sim 84,000 \text{ Da} \) structure may be an IMPDH-II dimer missing a large portion of both flanking domains. A similar \( \sim 86,000 \text{ Da} \) IMPDH-II structure has been found in \textit{Aerobacter aerogenes}.\textsuperscript{20} If we consider the monomer as the primary analyte of interest (instead of the tetramer), clustering may also explain the \( \sim 84,000 \text{ Da} \) species. Multimers can reflect real species in solution, or they can be formed in plume reactions in the plasma just above the target surface. The \( \sim 84,000 \text{ Da} \) size corresponds to the possible cluster ion \( [3M + 2H]^2^+ \) where, considering a monomeric subunit mass (\( \sim 55,000 \text{ Da} \)), the resulting species would be detected at a size of 83,700 Da.

Aside from clustering, the sample may also form an adduct ion with molecules of the matrix. For larger proteins, mass measurement accuracy may be compromised by the inability to distinguish between \([M + H]^+\) ions and adducts formed between the sample and matrix. The resolution for low-mass ions is much better than that for high-mass ions. This effect is the result of the spread of the initial kinetic energy of the ions. The high yield of low-mass ions saturates the detector, which results in lower efficiency for the high-mass ions because the detector can not recover quickly enough. Also, salt concentrations larger than 0.1 mM generally result in a progressive decrease in mass spectra signal (i.e. as salt concentration increases, signal decreases). The protein dilution buffer used in these studies contains 50 mM NaCl, thus the spectra measurements, in particular for the tetrameric IMPDH-II species, are likely underestimated.

A characteristic effect of imposing energy on a multi-subunit protein species is that, if the intensity level is great enough, it may break apart the non-covalent bonds that
hold protein subunits together. In MALDI analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, usually an UV-absorbing weak organic acid, which strongly absorbs the laser light energy and causes the analyte to vapourize. The matrix plays a key role since it absorbs the energy ("damage") to a larger extent than the analyte, thus minimizing damage to the original sample. Further, since successive spectra can be obtained from a single spot on the sample target, it can be concluded that this technique is non-destructive. If 'breaking' were occurring, one would expect to see an increase in the relative abundance of all size populations as the concentration of the sample increases. Four masses were observed that corresponded to multiple subunit sizes of IMPDH-II up to its native tetrameric structure, and a fifth size (~ 84,000) that may be an IMPDH-II dimer missing both flanking domains, or a multiply charged species. While the amount of all the multimeric species slightly increased with an increase in concentration, the monomeric species decreased by nearly a third instead of increasing (i.e. 651 at 0.10 mg/ml to 261 at 2.0 mg/ml). This supports the assumption that the 'breaking event' is not a major factor. There may, however, be a different general effect occurring with a change in concentration. As the concentration of IMPDH-II increased, the differences in the relative populations of monomer, dimer, trimer, and tetramer corresponded to a typical shift in equilibrium that the components of a reversible self-associating system would experience with such a change in concentration. Generally, with increasing concentration, the amount of tetramer, trimer, and dimer would increase and the amount of monomer would decrease. Mass spectrometry has proved to be an effective tool in characterizing self-associating systems. Recently, this technique was
used to report that the human homologue of the *Drosophila* discs large tumour suppressor protein (hDlg) self-associates into dimeric and tetrameric species in solution.²⁵

### 2.5.3 Non-Denaturing Polyacrylamide Gel Electrophoresis

A non-denaturing gel system separates native proteins based not only on protein size, but also on shape and charge. For this reason, accurate estimation of molecular weight can be challenging for some proteins by this method, and identification of a specific protein in a mixture by its molecular weight without a specific detection method is very difficult (although improved weight determination can be achieved by using gradient gels).²⁶ Thus, using a non-denaturing gel system is recommended only if one needs to analyze native proteins rather than denatured ones, particularly if the biological activity (i.e. enzyme activity) of a protein needs to be retained for subsequent steps. In non-denaturing gel systems, the choice of buffer pH may depend on the isoelectric points of the protein(s) being examined. When examining a single protein, however, it is not as complicated or imperative to find a suitable pH since only one isoelectric point needs to be considered. Aside from charge, a non-denaturing system also separates proteins in terms of shape and size. This approach is used typically to assess heterogeneous mixtures of proteins; in the present study, however, we are interested in the population distribution for a homogeneous mixture. Silver staining was used to detect the separated protein bands. This method depends on the reduction of ionic to metallic silver to provide metallic silver images. Ionic silver binds to sites in the gel occupied by protein and there is selective reduction of silver ions to metallic silver at gel sites where protein
bands are present. Silver staining is among the most sensitive staining techniques as it can detect protein quantities as low as 1 ng.\textsuperscript{27}

Non-denaturing PAGE performed on 2.0, 0.10, and 0.02 mg/ml IMPDH-II revealed the presence of several protein bands. At 2.0 mg/ml, one large, dark, and diffuse band was detected at an approximate molecular weight of 200,000 Da. It appeared that this concentration was too high to distinguish between constituent bands. At 0.10 mg/ml, the large band found in Lane 1 was condensed to one dark band at \( \sim 220,000 \) Da with a very diffuse streak to \( \sim 110,000 \) Da. At 0.02 mg/ml, there were three bands: a faint band at \( \sim 220,000 \) Da, a faint band at \( \sim 110,000 \) Da, and finally a clear and dense band at \( \sim 55,000 \) Da was detected. These bands correspond to the IMPDH-II tetramer, dimer, and monomer and clearly reveal the ability of non-denaturing PAGE to separate a homogeneous protein mixture into its constituents. Thus, in lanes 1 and 2, the intensity of the bands at \( \sim 220,000 \) (tetramer) and \( \sim 110,000 \) Da (dimer) decreased with decreasing concentration. Further, at these concentrations, the relative amount of monomer was not sufficient to be detected by silver staining. In lane 3 (0.02 mg/ml IMPDH-II), however, the presence of the monomer is clear along with a corresponding decrease in intensity of the dimer and tetramer bands. The presence of three bands suggests the likely equilibrium between IMPDH-II multimers in solution.

These results, combined with those observed by circular dichroism and mass spectrometry, confirm that at low concentrations IMPDH-II exists in a dynamic equilibrium of multimers from monomers to tetramers. This follows the principles governing reversible self-associating systems, whereby the composition, and therefore the average molecular mass, are uniquely determined by the concentration according to
the law of mass action. One possibility for the mechanism of IMPDH-II self-association is that monomers come together to form dimers, which then associate to form tetramers. The dimers can also combine with other monomers to form trimers, although combining with other dimers to form tetramers may be a more favourable step. Conversely, upon dissociation tetramers would most likely break into dimers and then monomers. This would drive the equilibrium to favour monomers, dimers, and tetramers, with minimal amounts of trimers in solution and thus, would explain the presence of three bands by non-denaturing PAGE at low concentrations. Although the dimer may be the most stable intermediate, small amounts of trimers would undoubtedly be formed during subunit association or dissociation, and this is confirmed by mass spectrometry. This technique also reported, however, that the tetramer was the smallest size population present instead of the trimer. As discussed, due to technical limitations associated with mass spectrometry the relative abundances of the tetramer are underestimated and thus, the concentration-dependent equilibrium proposed between IMPDH-II monomers, dimers, and tetramers is probable.

The presence of NaCl in our protein dilution buffer may have also helped to achieve this equilibrium. At high protein concentrations (≥ 2.0 mg/ml), the absence of a reducing agent may result in aggregation, however, at low concentrations aggregation is not likely and tetrameric structures may dissociate into constituent monomers. Furthermore, the presence of ions may help to stabilize residues normally participating in non-covalent interactions existing at the interface between adjacent subunits. The dependence of this equilibrium on degree of ionic content is not clear. All experiments were performed with NaCl concentrations near physiological levels (~ 150 mM), except
for mass spectrometry, which used a dilution buffer containing 50 mM NaCl. Regardless of the salt level, all results showed the presence of an equilibrium effect. Thus, the major factor for this observation appears to be protein concentration with additional stabilization provided by the presence of salt.

This type of reversible self – associating behaviour in protein systems has been well documented. Furthermore, the equilibrium observed here (monomer, dimer, tetramer) is much more common than that including the trimer as a major intermediate. It has been found that the dimer is a stable intermediate in the dissociation of tetrameric malate dehydrogenase to monomers at low pH. Circular dichroism spectra on native alpha s1-casein show that ionic strength and protein concentration lead to increases in the degree of self – association of the protein from dimers to higher oligomers. Self – association studies of the ATPase ClpB from Escherichia coli have found that the monomeric form predominates at low protein concentration (0.07 mg/ml), while an oligomeric form is highly populated at concentrations greater than 4 mg/ml. Phosphoglycerate mutases from different sources also exist as tetramers, dimers, and monomers at low concentrations. Dissociation of the fully active tetramer of rabbit skeletal muscle phosphofructokinase occurs in three kinetic phases: (1) dissociation of tetramer to dimer; (2) dissociation of dimer to monomer and; (3) conformational change of the monomer. The crystal structures of pyruvate decarboxylase from the yeast Saccharomyces uvarum and Saccharomyces cerevisiae have provided details about monomer assembly to form dimers and dimer assembly to form tetramers. Other systems have exhibited similar equilibrium characteristics.
Our results demonstrate that a dynamic equilibrium between IMPDH-II monomers and tetramers exists with the dimer as the intermediate species. We have shown that the IMPDH-II multimer equilibrium is concentration-dependent with an increasing shift towards the monomeric species with decreasing IMPDH-II concentration. Furthermore, it is believed that the presence of ions in solution helps to achieve this equilibrium by stabilizing monomeric and other intermediate IMPDH-II structures.

2.5.5 IMPDH-II Activity Assays

In order to understand a variety of biological processes it is often necessary to identify and characterize specific proteins using electrophoretic-based techniques. Electrophoresis performed using one-dimensional gels is used to separate and identify proteins within a mixture. Electroblotting or Western transfer is a powerful second-dimension technique whereby the proteins separated in a one-dimensional gel are electrophoresed out of the gel and onto a solid phase where they can be exposed to various detection methods for specific identification. An effective method of assessing enzyme activity is to expose a protein or protein mixture to non-denaturing PAGE and then electroblotting the separated sample to a transfer membrane. Here, the protein band of interest can be excised and then used in a conventional enzyme activity assay. PVDF membranes are generally better to use than nitrocellulose membranes since they utilize strong hydrophobic forces to bind protein molecules. This is particularly important for this study since it is critical that the IMPDH-II monomers are held tightly so as to not allow reassembly of the IMPDH-II monomers into the tetrameric form at the membrane.
surface. The technique described here has been used to study the macromolecular self-association of ADP-ribosyltransferase and its correlation with enzymic activity.\textsuperscript{43}

In this study, control activity assays were run using typical assay enzyme quantities (10 \( \mu \)g) and quantities used in the non-denaturing PAGE analysis (0.60 \( \mu \)g (30 \( \mu \)l loaded of 0.02 mg/ml IMPDH-II)). Each PVDF membrane section corresponding to the 220,000, 111,000, and 55,000 Da protein bands showed some degree of activity. Interestingly, the monomer showed the greatest production of NADH, then the dimer, and then the tetramer suggesting that the more active protein was in the monomer band at 0.02 mg/ml. After 60 minutes, the total production of NADH by the different IMPDH-II protein bands at 0.02 mg/ml was approximately 4 times that of the same total amount of whole protein. Furthermore, assuming the Western transfer was efficient, after 60 minutes the monomeric protein band in lane 3 (0.60 \( \mu \)g) alone showed approximately twice the production of NADH than the control assay using the same amount of whole protein (i.e. 0.60 \( \mu \)g of monomers, dimers, and tetramers). While there is no question that the IMPDH-II monomer does retain activity, it is not clear as to why its activity was so high, along with the total activity of the protein bands.

As with non-denaturing PAGE, a Western transfer involves electrophoretic transfer of the protein from the polyacrylamide gel to the PVDF membrane. This migration is based on charge and results in a specific alignment of the protein on the membrane. The active site of IMPDH-II lies in a large negative electrostatic region and is directly opposite a large positive electrostatic region (Refer to Chapter 4; Figure 4.4.3). Thus, while the protein is bound to the membrane, the active site may align itself with the membrane surface. This orientation of the active site would undoubtedly facilitate the
activity assay, as the active site is readily exposed on the membrane and available for reaction with substrates in solution. This theory of charge induced orientation is supported by the fact that the same quantity of IMPDH-II deposited on a PVDF membrane by dot blotting resulted in activity that was considerably lower. Since dot blotting transfers protein to a membrane by a vacuum, there is no expectation of orientation. This may explain why the activity observed for IMPDH-II transferred to PVDF membranes by Western transfer is higher than that by dot blotting. It still remains unclear, however, as to why the activity of IMPDH-II, when transferred by Western transfer is higher than when the assay is performed in free solution. Nevertheless, it has been clearly demonstrated that the immobilized IMPDH-II monomer does possess enzymatic activity. These results are similar to those found for IMPDH from Aerobacter aerogenes.42

The reassembly of IMPDH-II monomers and/or dimers into tetramers is an important issue to discuss. During electrophoretic transfer from the gel to the PVDF membrane, it is very unlikely that tetramer reformation occurs during the transfer process as the gel and PVDF membrane are held in very close proximity. Furthermore, while on the PVDF membrane, protein molecules are held to the surface by strong hydrophobic bonds, preventing reassembly of IMPDH-II molecules on the membrane surface. Finally, release of IMPDH-II monomers and/or dimers from the PVDF membrane during the activity assay may result in solution reassembly into the tetrameric form. Spectroscopic analyses of the buffer solution did not reveal the presence of IMPDH-II confirming that it is held strongly to the PVDF membrane surface. Thus, tetramer re-formation is not
occurring during the aforementioned experiments affirming that monomeric IMPDH-II does retain activity.

Each IMPDH-II monomer possesses an active site with the binding pocket at the interface between adjacent subunits in the tetrameric structure (Refer to Chapter 1; Figure 1.1.4). The protein-ligand interactions within the IMP binding region are completely contributed by the active site monomer. While the protein-ligand interactions within the NAD binding region are also dominated by the active site monomer, a few stabilizing interactions exist that involve residues from adjacent monomers. In the human form, the adenosine end of the dinucleotide analogue SAD is bound between the α3 helix – β3 sheet junction and participates in many interactions with the active site monomer. Here, the adenine ring is tightly stacked between Phe-282 and His-253. Furthermore, there are interactions between the adenine amino group and the side chain of Thr-252, and the adenosine diphosphate component and Ser-275 and Ser-276. In this position the adenine ring also participates in interactions with residues from the βC – βD sheet junction of the adjacent monomer. These interactions include two direct hydrogen bonds between the ribose hydroxyls and Gln-469, and electrostatic contact between the adenine base and Thr-45 (Figure 2.5.2.). Since Gln-469 and Thr-45 are residues contributed by a neighbouring monomer, they would be absent in the monomeric active site.

Although cofactor binding may be decreased in the IMPDH-II monomer, we would expect to see some retention of activity for the following reasons. First, the majority of the residues interacting with IMP and NAD belong to the active site monomer. Secondly, upon NAD binding the adenine ring of the cofactor is stacked between the Phe-282 and His-253 residues of the active site monomer in a very tight
conformation. Thirdly, binding of the cofactor causes the putative sections of the active site flap, seen in the human form, to make contact with the adenosine portion of the dinucleotide and provides the major stabilizing force around the active site. These data strongly suggest that there are sufficient stabilizing interactions between monomeric IMPDH-II, IMP, and NAD⁺ to enable enzymatic reactions to take place.

Figure 2.5.2. Interactions involving the dinucleotide analogue adenine ring. Participating residues are shown in white and outlined by van der Waals surfaces. The adenine ring is tightly bound between Phe-282 and His-253, and participates in two direct hydrogen bonds between the ribose hydroxyls and Gln-469, and an edge-on contact between the adenine base and Thr-45. Gln-469 and Thr-45 are residues contributed by an adjacent monomer. Electron density is also observed between the adenine amino group and the side chain of Thr-252.

The fact that the IMPDH-II monomer shows slightly greater activity compared to the dimer and tetramer presumably reflects the relative amounts of each species at 0.02
mg/ml, however, this may also be related to the negative cooperativity associated with substrate binding among monomers in the tetrameric structure. Similar results have been reported for the tetrameric enzyme glyceraldehyde-3-phosphate dehydrogenase.
2.6 CONCLUSION

The present study has demonstrated that IMPDH-II exists as a reversible, self-associating system, with the predominant equilibrium between monomers, dimers, and tetramers. Although X-ray diffraction has revealed the structural details of an active tetrameric form, we now have evidence that monomeric IMPDH-II retains sufficient structure to elicit activity.
2.7 REFERENCES

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CHAPTER 3

**In Situ Formation of Supported Planar Bilayers by Atomic Force Microscopy**

3.1 OVERVIEW

Owing to their ease of fabrication, and controllable chemical and structural characteristics, supported planar bilayers (SPBs) are ideal for applications involving model membrane systems. We have created a protocol that will allow the *in situ* formation of large and molecularly smooth SPBs as substrates for investigating the structure of adsorbed proteins by atomic force microscopy (AFM). By interfacing a flow-through injection system with the fluid cell of a scanning probe microscope, the formation of L-alpha-phosphatidylserine, source bovine brain (PS), 1,2-dimyristoyl-phosphatidylcholine (DMPC), and 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP) bilayers was observed, including a previously undetected structure and dynamic event. *In situ* imaging revealed that SPB assembly occurs via a two-step process involving island formation and spreading, and lateral reorganization during island fusion. Simultaneous height and phase imaging also revealed the presence of a ~ 1 nm thick conformationally distinct overlayer on both the neutrally charged DMPC (~ 4.8 nm thick) and positively charged DMTAP (~ 4.5 nm thick) bilayers. Differences in the stability of the overlayer between the two lipid systems suggests that this is a general assembly phenomena governed by a subtle balance between electrostatic charge stabilization between the lipid headgroups and the enthalpic costs associated with exposure of the hydrophobic lipid tails. The effects of ion concentration on the formation of the overlayer support this theory. This protocol provides an ideal approach for not
only characterizing the structure of soluble ligands adsorbed to SPBs and elucidating protein-protein interactions in real-time, but also studying the dynamics of bilayer formation and self-assembly.
3.2 MATERIALS

Lyophilized lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Mica sheets were kindly provided by Dr. Eliot Chaikof at Emory University. Magnetic specimen preparation disks were purchased from Ted Pella Incorporated (Redding, CA, USA). Silastic brand medical grade tubing (1/16” ID, 1/8” OD, Lot Number 72568) was purchased from Fisher Scientific Company (Pittsburgh, PA, USA). Oxide-sharpened silicon nitride V-shaped AFM cantilevers were purchased from Digital Instruments (Model DNPS – 120 μm; Santa Barbara, CA, USA). Water was distilled and deionized using Millipore Milli-Q UF Plus (Bedford, MA, USA) at 18 MΩ resistance. All other reagents were of the highest analytical grade.
3.3 METHODS

3.3.1 Vesicle Suspension Preparation

Lyophilized PS, DMPC, and DMTAP phospholipids were dissolved in chloroform to a final concentration of 10 mg/ml, divided into 50 µl aliquots, and stored at -20 °C until needed. Vesicle suspensions were prepared by transferring the thawed lipid stock aliquots into a 20 ml threaded-neck Kimax test tube, and the solvent removed by rotary evaporation at 25°C. The resulting opaque lipid film was dried under vacuum for approximately one hour and re-suspended in 500 µl of pH 8.0 PBS buffer (150 mM NaCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄ unless otherwise specified) to yield a ~1 mg/ml solution and transferred to a 7 ml scintillation vial. The DMPC vesicle suspensions were subjected to 5 freeze/thaw cycles alternately in dry ice/acetone and room temperature water baths, and resuspended by vortex centrifugation. All suspensions were used immediately.

3.3.2 Supported Planar Lipid Bilayer Formation

To prepare the supported planar bilayers, freshly cleaved mica, previously affixed with epoxy to a steel AFM sample puck, was sealed in a combination contact / TappingMode™ AFM fluid cell (Digital Instruments; Santa Barbara, CA, USA). Silicone tubing was fitted to the drain port on the fluid cell with the inlet port outfitted with a polypropylene plastic Y-connector. This connector allows the exchange of fluids within the cell without disassembly or introducing air into the chamber. One end of the connector contains the imaging solution, in this case pH 8.0 PBS buffer, and the other
end serves as an inlet for new reagents. After obtaining a reference image of the mica substrate in buffer, approximately 500 µl of the lipid suspension of interest was introduced into the AFM fluid cell and allowed to stand for ~ 45 minutes. It was noted that the volume defined by the fluid cell and O-ring is ~ 200 µl, thus ensuring complete replacement of the buffer solution with the lipid solution of interest. Prior to imaging, the fluid cell was thoroughly flushed with pH 8.0 PBS buffer to remove excess lipid.

3.3.3 Atomic Force Microscopy

Solution TappingMode™ atomic force microscopy (TMAFM) was performed on a Digital Instruments Nanoscope IIIA MultiMode scanning probe microscope (Digital Instruments; Santa Barbara, CA, USA) using 120 µm oxide-sharpened silicon nitride DNP and DNP-S V-shaped cantilevers installed in a combination contact / TappingMode™ liquid flow cell. The cantilever tips were pre-treated for ~ 30 minutes with UV irradiation to remove adventitious organic contaminants. All TMAFM images were acquired using the E scanning head, which has a maximum lateral scan area of 14.6 µm x 14.6 µm, at tip scan rates from 1.50 – 2.50 Hz and cantilever drive frequencies of ~ 7-9 kHz. All images were captured as 512 x 512 pixel images and low-pass filtered. Feature size and volumes were calculated using the Digital Instruments Nanoscope software (version 4.21), and shareware image analysis program, NIH-Image (version 1.62).
3.3.4 Electron Microscopy

For negative staining, carbon-coated pioloform grids were floated on aqueous solutions of phospholipids (0.5 mg/ml). After grids were blotted and air-dried, the samples were stained with 1% (w/v) phosphotungstic acid. All electron microscopy imaging was performed using a Hitachi 7000 electron microscope operating at 75 kV. Images were captured on Kodak Electron Microscope Film, # 4489.
3.4 RESULTS

3.4.1 Supported Planar Lipid Bilayer Formation

3.4.1.1 L-Alpha-Phosphatidylserine

Absorption of PS phospholipid vesicles from solution onto mica resulted in the formation of large bilayer islands, which eventually fused to form large, planar SPBs (Figures 3.4.1A and 3.4.1C). Close inspection of the SPB surfaces revealed that they were molecularly smooth and defect free. The bilayers had an approximate height of 4.9 ± 0.5 nm (Figure 3.4.1D), which is close to an expected value of 5 nm. Previous studies have reported the existence of a 1 - 3 nm thick fluid buffer layer between the bottom leaflet of the SPB and the supporting mica.\(^1\)\(^-\)\(^3\) Furthermore, slight discrepancies in the observed feature height, as determined by atomic force microscopy, have been attributed to electrostatic interactions between the tip and sample during imaging.\(^4\) Phase detection provided an additional high level of image contrast between the mica and bilayer (Figure 3.4.1B). Extended duration imaging performed over a 47 minute period revealed gradual expansion of the bilayer (Figures 3.4.1A and 3.4.1C). The cumulative increase in SPB area was \(~ 4,636,382\) nm\(^2\) over this time period, corresponding to a growth rate of \(~ 1,644\) nm\(^2\)/sec respectively. Assuming that PS phospholipids have a head group area of \(~ 0.60\) nm\(^2\),\(^5\) this translates to an SPB growth rate of \(~ 2,740\) molecules/sec. Extensive large area scanning did not reveal any evidence of tip-induced rearrangement or reorganization of the bilayer.
Figure 3.4.1. *In situ* tapping mode AFM images acquired in pH 8 PBS buffer of a PS bilayer on mica. AFM height image showing a discontinuous section of bilayer at (A) time = 0 minutes and at, (C) time = 47 minutes. (B) AFM phase imaging of (A) indicates phase contrast between the mica, (1), and bilayer, (2). (D) Section analysis across a – a’ in (A) reveals an approximate bilayer height of 4.9 ± 0.5 nm. Image sizes: 10 μm x 10 μm. Images acquired at a scan rate of 1.80 Hz. Scale bar: 2 μm.
3.4.1.2 1,2-Dimyristoyl-Phosphatidylcholine

Adsorption of DMPC phospholipid vesicles onto mica resulted in the formation of large, molecularly smooth, and defect-free SPBs (Figure 3.4.2A), with an approximate thickness of 4.8 ± 0.5 nm (Figure 3.4.2D). Close inspection by simultaneous phase (Figure 3.4.2B) and height contrast imaging revealed patches of a 1 ± 0.1 nm thick overlayer on top of the bilayer. There was no evidence of this structure on any exposed mica surfaces. Extended duration imaging performed over a 55 minute period revealed gradual dissipation of the ~1 nm thick overlayer with a concomitant expansion of the underlying bilayer. (Figure 3.4.2C). The cumulative decrease in overlayer area of ~680,000 nm² was accompanied by an increase in SPB area of ~150,000 nm² over this time period, corresponding to area dissipation and growth rates of 206 nm²/sec and 45 nm²/sec respectively. DMPC phospholipids have a head group area of ~0.50 nm². This translates to an overlayer dissipation rate of ~412 molecules/sec and an SPB growth rate of 90 molecules/sec. In this case, the rate of overlayer dissipation is ~four and a half times that of bilayer growth. Extensive large area scanning did not reveal evidence of tip-induced rearrangement or reorganization of the bilayer.
Figure 3.4.2. *In situ* tapping mode AFM images acquired in pH 8 PBS buffer of a DMPC bilayer on mica. AFM height image showing a discontinuous section of bilayer, with the faint appearance of a second overlayer on the bilayer surface, at (A) time = 0 minutes and at, (C) time = 55 minutes. The arrows show corresponding bilayer holes. (B) AFM phase imaging of (A) indicates phase contrast between the mica, (1), bilayer, (2), and overlayer, (3). (D) Section analysis across a – a’ in (A) reveals an approximate bilayer height of 4.8 ± 0.5 nm and overlayer height of 1.0 ± 0.1 nm. Image sizes: 5 μm x 5 μm. Images acquired at a scan rate of 1.97 Hz. Scale bar: 1 μm.
3.4.1.3 1,2-Dimyristoyl-3-Trimethylammonium-propane

Absorption of the DMTAP phospholipid vesicles onto mica also resulted in the formation of large, planar, and molecularly smooth SPBs (Figure 3.4.3A). Section analyses revealed that these molecularly flat bilayers had an approximate thickness of 4.5 ± 0.5 nm (Figure 3.4.3D). As with DMPC, close inspection by simultaneous phase (Figure 3.4.3B) and height contrast imaging revealed patches of a 1 ± 0.1 nm thick overlayer on top of the bilayer. There was no evidence of this structure on any exposed mica surfaces. Extended duration imaging performed over a 75 minute period revealed gradual dissipation of the ~1 nm thick overlayer with a concomitant expansion of the underlying bilayer. (Figure 3.4.3C). The cumulative decrease in overlayer area of ~4,190,000 nm² was accompanied by an increase in SPB area of 2,142,000 nm² over this time period, corresponding to area dissipation and growth rates of 1,025 nm²/sec and 500 nm²/sec respectively. DMTAP phospholipids have a head group area of ~0.70 nm². This translates to an overlayer dissipation rate of ~1,465 molecules/sec and an SPB growth rate of 715 molecules/sec. Remarkably, the rate of overlayer dissipation is approximately twice that of bilayer growth, which strongly suggests direct incorporation of the overlayer molecules into the growing bilayer (vide infra). Extensive large area scanning did not reveal evidence of tip-induced rearrangement or reorganization of the bilayer.
Figure 3.4.3. *In situ* tapping mode AFM images acquired in pH 8 PBS buffer of a DMTAP bilayer on mica. AFM height image showing a discontinuous section of bilayer, with the faint appearance of a second overlayer on the bilayer surface, at (A) time = 0 minutes and at, (C) time = 75 minutes. (B) AFM phase imaging of (A) indicates phase contrast between the mica, (1), bilayer, (2), and overlayer, (3). (D) Section analysis across a – a’ in (A) reveals an approximate bilayer height of 4.5 ± 0.5 nm and overlayer height of 1.0 ± 0.1 nm. Image sizes: 5 μm x 5 μm. Images acquired at a scan rate of 1.70 Hz. Scale bar: 1 μm.
Absorption of the DMTAP phospholipid vesicles onto mica in the presence of 200 mM NaCl also resulted in the formation of large and uniform SPBs (Figure 3.4.4A). Section analyses revealed that these molecularly flat bilayers had an approximate thickness of 4.5 ± 0.5 nm (Data not shown). As expected, height and phase (Figure 3.4.4A and 3.4.4B) imaging revealed patches of a 1 ± 0.1 nm thick overlayer on top of the bilayer. There was no evidence of this structure on any exposed mica surfaces. Extended duration imaging performed over a 120 minute period revealed the gradual growth of the ~1 nm thick overlayer with a concomitant expansion of the underlying bilayer. (Figure 3.4.4A and 3.4.4C). The cumulative increase in overlayer area of ~53,210,000 nm² was accompanied by an increase in SPB area of 1,002,000 nm² over this time period, corresponding to overlayer and bilayer growth rates of 443,395 nm²/sec and 835 nm²/sec respectively. DMTAP phospholipids have a head group area of ~0.70 nm². This translates to an overlayer growth rate of ~633,420 molecules/sec and an SPB growth rate of 1,195 molecules/sec. Extensive large area scanning did not reveal evidence of tip-induced rearrangement or reorganization of the bilayer.
Figure 3.4.4. *In situ* tapping mode AFM images acquired in pH 8 PBS buffer (200 mM NaCl) of a DMTAP bilayer on mica. (A) AFM height image showing a large bilayer island with the faint appearance of a second overlayer on the upper left and lower left sections of the bilayer surface. (B) Corresponding phase image to (A) indicates 3 distinct levels of phase separation between the mica (1), bilayer (2), and overlayer (3). AFM height (C) and phase (D) image on the same bilayer island 2 hours later showing the gradual growth of the overlayer across the bilayer surface to the right. Image sizes: 12.5 μm x 12.5 μm. Images acquired at a scan rate of 1.70 Hz. Scale bars: 2.5 μm
3.5 DISCUSSION

3.5.1 Supported Planar Bilayer Formation

These *in situ* TMAFM experiments performed on PS, DMPC, and DMTAP SPBs formed by lipid vesicle fusion resulted in the formation and growth of ~5 nm thick large, flat, and texture-free bilayer islands. The formation of SPBs by vesicle fusion has been proposed to follow a three-step process, and was recently experimentally verified. In this model, lipid vesicles, either as unilamellar or multilamellar structures, adsorb to a solid interface where they may rupture spontaneously, fuse with an adjacent vesicle prior to rupturing, or collapse into a bilayer structure (Figure 3.5.1). This process is driven by the relative magnitudes of the adhesive interactions between the vesicle and solid substrate and the unfavourable energies associated with the curvature of the vesicle walls. The process of lipid fusion and spreading can be related to the relative size of the individual planar bilayer disks with spreading being facilitated once a critical vesicle rupture radius has been achieved. Such a model would be akin to a critical nuclei size required for crystal growth. At dimensions less that the critical rupture radius, spreading is not favoured whereas fusion occurs preferentially for larger vesicles. This model does not account for the presence of bilayer sheets. Bilayer sheets would fuse directly with the solid support from solution to form intact supported bilayers. This process can occur in tandem with the model of vesicle fusion proposed by Reviakine and Brisson. The data from this study are consistent with these models, as the growth of large two-dimensional islands of PS, DMPC, and DMTAP were routinely resolved, with subsequent entrainment of smaller stationary lipid islands with a minimum diameter of approximately 50 nm if viewed as a planar disk (data not shown). The result is the formation of large,
membrane-mimetic substrates that closely resembled the physical and mechanical properties of bilayer surfaces.\textsuperscript{11,12} As opposed to SPBs formed by Langmuir-Blodgett (LB) techniques, the SPBs formed here are well suited for studies that require a substrate with a certain degree of bilayer fluidity. Certainly, this would be paramount for studies that focus on characterizing intrinsic bilayer properties.

![Diagram of vesicle behavior](image)

**Figure 3.5.1.** Adapted from Reviakine and Brisson.\textsuperscript{10} (A) Lipid vesicles adsorb to mica with a radius \((r, R)\) which is either less than or greater than the rupture radius \((R)\). (B) Surface-bound vesicles with a radius less than the rupture radius fuse with other similar vesicles. (C) Once the rupture radius has been reached, either by initial adsorption or inter-vesicle fusion, rupture occurs which leads to the formation of single bilayer islands.

The \textit{in situ} TMAFM experiments performed on both DMPC (neutral) and DMTAP (positive) lipid bilayer surfaces also revealed the presence of a metastable ~1 nm thick overlayer. Contiguous with the underlying bilayer, this thin film was never found on the underlying mica surface, and its relative stability was found to be highly
dependent on the lipid structure and surrounding electrolyte concentration. Although resolvable by height imaging, phase detection provided much higher level of image contrast between the mica, bilayer, and the overlayer. Phase imaging measures the relative phase shift between the applied and detected tip oscillation during TMAFM imaging, and thus can provide information regarding intrinsic local surface modulus and viscoelasticity. In fluid, the observed phase shift presumably reflects differences in the amount of energy dissipated during tip contact with the surface and may correlate with specific adhesive interactions between the tip and surface, with the degree of phase shift increasing with increasing adhesion and energy dissipation.

In both bilayer systems, the transient overlayer exhibited a different level of phase lag relative to the reference mica substrate and fully formed lipid bilayer (refer to Results; Figure 3.4.1B and 3.4.3B). As phase imaging of multi-island lipid structures (data not shown) provides image contrast only at the bilayer edges where there is a transient phase shift due to interaction between the scanning tip and the topographical feature, akin to friction imaging which provides heightened contrast at feature edges due to increased torque on the scanning tip during contact imaging, these data strongly suggest that the overlayer is not an imaging artifact or a disordered lipid bilayer. Rather, we propose that this phase shift, and corresponding topographical difference of ~ 1 nm, is due to a monolayer of disordered, likely collapsed, lipid molecules. Although the orientation of the lipid molecules (head-up or head-down) cannot be definitely assigned, it is reasonable to presume that the overlayer is arranged such that the lipid headgroups are down and associated with the headgroups of the bilayer. In this model, the AFM tip is providing height and phase contrast consistent with interactions between the scanning tip and the
hydrophobic acyl lipid tails. In this orientation, the tails are likely collapsed with the lipid monolayer anchored via charge-charge interactions with the supporting bilayer. Such an inverted structure has been reported for LB films of pulmonary surfactant.17

3.5.2 Overlayer Formation Motif

The mechanism of formation of these overlayers may be explained in terms of the structure of micelles. Electron microscopy revealed that the DMPC vesicle suspension was mainly composed of micelles, and the DMTAP vesicle suspension was composed of a mixture of planar lipid sheets and micelles (data not shown). This suggests that the initial formation of the underlying SPB is accomplished by the fusion of micelles as described above by Reviakine and Brisson, as well as the direct fusion, from solution, of intact bilayer sheets onto mica. As the growing bilayer sheet covers the surface of the mica, the micelles from the suspension may also begin to adsorb to the surface of the bilayer. Under normal circumstances, these vesicles would follow the Reviakine and Brisson model and eventually rupture or collapse and form a double bilayer region. Rather than collapsing and to form a second bilayer sheet, however, these micelles may self-stabilize at the bilayer surface. This process may be driven by preferential surface stabilization between the vesicle and bilayer headgroups, which is a consequence of the electrolyte concentration. For charged phospholipids, this effect would be magnified as the presence of counterions in solution may contribute added stability between charged head groups. Favorable head group stabilization of the micelles would eventually drive opening of the micelle to form a monolayer on the bilayer surface (Figure 3.5.2).
Figure 3.5.2. Schematic model of a possible lipid overlayer binding motif. (A) Bilayer formation is accomplished by both fusion and collapse of micelles on mica and the direct fusion of large bilayer sheets. (B) Over time, the micelles from the suspension adsorb to the bilayer surface. (C) Here, they are stabilized by favourable head group–head group interactions and bridging ions, which eventually cause the micelle to unroll into a monolayer structure (D).

3.5.3 Surface Reorganization

Extended-duration *in situ* AFM imaging on DMTAP and DMPC SPBs in 150 mM NaCl revealed a gradual expansion of the bilayers accompanied by a more rapid disappearance of the overlayer. Dissociation appeared to preferentially occur at the periphery of the overlayer, although in the case of the DMTAP bilayers, small ~75 nm diameter holes did form in the bulk of the overlayer. These holes subsequently acted as nucleation sites for further overlayer dissociation. In both cases, the rate of overlayer disappearance was substantially higher than that for the growth of the supporting bilayer. It should be noted that in these experiments, there was no evidence suggesting that the
scanning tip played a significant role in promoting these reconstruction phenomena. These results strongly suggest that the overlayer molecules are in an unstable structural motif and that this strain is relieved by surface reorganization. Based on these observations, various mechanisms for this reassembly process may be posed. The overlayer molecules may undergo an in-plane flip to reinsert their hydrophobic tails into the bilayer. This process may be facilitated by the highly fluidic nature of the bilayers formed in the present study. This process results in reinsertion only into the upper leaflet of the bilayer, however, and will, in time, result in an energetically unfavourable structure due to upper leaflet curvature. Gradual unrolling and expansion of the bilayer may accommodate this strain. Transbilayer exchange may occur wherein lipid molecules adsorbed to the upper leaflet may diffuse though the bilayer to the lower leaflet through a transient lipid pore. Such a mechanism may be facilitated by the high lateral fluidity of the bilayers when prepared by simple vesicle fusion. It is clear, however, that the energetic cost associated with this process is high, and there is an accompanying increase in diffusion time relative to lateral motion through the bilayer. In this case, typical exchange times can be on the order of minutes to days and are dictated more by the structure of the lipid headgroup. We discount this possibility in the case of bilayers supported by a solid substrate since they do not truly mimic a fluid bilayer environment.

These results suggest that the overlayer molecules, which we contend are lipid molecules tethered by charge interactions between lipid headgroups, dissociate either by resorption into the surrounding fluid or via lateral diffusion across the underlying bilayer, and are subsequently reincorporated into bilayer edges and defect sites (Figure 3.5.3). This model is in agreement with the observation that the overlayer receded away from the
bilayer edges during rearrangement. Although the possibility that the action of the scanning tip facilitates these processes cannot be discounted, this reassembly process occurred outside of the scanning window and there was no evidence of any tip-induced reorganization of either the overlayer or bilayer.

Regardless of the actual mechanisms of reorientation, the total amount of material in the imaging chamber remains constant during imaging since additional material was not added to the AFM fluid cell. Since mass must be conserved, and assuming that the major source of lipid is the bilayer and overlayer, there may be a link between the rate of disappearance of the overlayer and the rate of bilayer repair (or formation). If one assumes that all the overlayer molecules are reconstituted within the growing bilayer then on an area per headgroup basis, the rate of overlayer disappearance should be approximately twice that of the underlying bilayer. Perhaps coincidentally, this was the case for the DMTAP bilayers (150 mM NaCl) where the approximate overlayer dissociation rate was twice that of the underlying bilayer. This was not the case, however, for the neutrally charged DMPC bilayers where the overlayer dissociation was significantly higher. While additional material was not added to the imaging chamber during imaging, after flushing out the bulk of the vesicle suspension a residual amount may remain in solution above the bilayer surface or between bilayer islands on the mica surface. This would account for the additional mass needed to fuel overlayer growth on DMTAP bilayers in 200 mM NaCl. It should be noted that the definition of "new bilayer" includes those regions formed by both lateral expansion of existing bilayer domains and areas of bilayer defect repair.
Figure 3.5.3. Schematic model of surface reorganization mechanisms. In (A), the overlayer molecules are associated in a head-to-head motif with the underlying bilayer. In this motif, the overlayer molecules are in an energetically unfavourable orientation and thus can reorganize themselves according to two possible paths. In path 1, the overlayer molecules can dissociate from the bilayer surface into the surrounding fluid as shown in (B1). Active lipid desorption from the bilayer surface is followed by rapid reintegration in (C1), either at the bilayer edges and defect sites, or at the bilayer surface in the original orientation. In path 2, the overlayer molecules can move via lateral diffusion across the underlying bilayer to the edges in (B2). At the edges, these inverted molecules can subsequently reincorporate themselves into the underlying bilayer (C2). As seen in (D), both mechanisms result in a decrease in the overlayer surface area with a concomitant increase in the bilayer surface area.
The differences between DMTAP and DMPC overlayer stability at 150 mM NaCl, and between DMTAP overlayer stability at 150 and 200 mM NaCl, may be accounted for when considering lipid head group charge and electrolyte concentration. As discussed, overlayer formation may be driven by preferential surface stabilization between the vesicle and bilayer headgroups. For charged phospholipids, this effect would be magnified as the presence of counterions in solution may contribute added stability between charged head groups. According to this model, positive DMTAP phospholipids would be held with a greater force at the head group–head group interface, and dissociate at a slower relative rate compared to bilayer growth. Conversely, at the same electrolytic concentration neutral DMPC phospholipids would be held less strongly, and dissociate at a faster relative rate compared to bilayer growth. Furthermore, the stability of the DMTAP overlayer should increase with increasing salt concentration due to the greater ability of stabilizing the head group charge interaction.

In the case of charged lipid suspensions, double-layer repulsive forces decay exponentially with a decay constant equal to the Debye screening length, which scales inverses with the square root of the electrolyte concentration \((C_o)\) as the following (for an aqueous solution with a 1:1 electrolyte):

\[
\text{Debye screening length (nm): } \frac{1}{\kappa} = \frac{0.304}{\sqrt{C_o}}
\]

Under our experimental conditions, the DMTAP bilayer and vesicles are similarly charged and would be unlikely to spontaneously self-associate due to the presence of significant double-layer forces; however, in the presence of high salt, the repulsive forces are moderated by the following:
\[ \frac{F}{\text{area}} = 64kTC_0 \Gamma_0^2 e^{-\kappa} \]

where \( \Gamma_0 = \tanh \left( \frac{Ze\phi_0}{4kT} \right) \)

From this expression, we can clearly see that the repulsive force per unit area is strongly dependent on the electrolyte concentration as:

\[ \frac{F}{\text{area}} \propto C_0^* e^{-\kappa} \quad \text{where} \quad \frac{1}{\kappa} = \frac{0.304}{\sqrt{C_0^*}} \]

\[ \Rightarrow \frac{F}{\text{area}} \propto C_0^* e^{\left( -\kappa \sqrt{C_0^*} \right)} \]

\[ \Rightarrow \frac{F}{\text{area}} \propto C_0^* e^{\left( -0.304 \sqrt{C_0^*} \right)} \]

This demonstrates that as the counterion concentration increases, the repulsive forces felt by the similarly charged species decreases. These theoretical considerations are in good agreement with our experimental SPB observations. At 150 mM NaCl, positive DMTAP overlayers dissipated at a slower rate than neutral DMPC overlayers. Furthermore, as the NaCl concentration increased from 150 mM to 200 mM, there was a change in DMTAP overlayer reorganization from slow dissipation to stabilized growth.

Recognizing the temporal limitations of AFM, the observed dissipation / growth rates are several orders of magnitude lower than the reported in-plane bilayer diffusion rates for lipids across a bilayer surface. It should be noted here that the typical residence time of lipid molecules within bilayers can be on the order of \(10^4\) seconds. This can be extrapolated to a residence time within the overlayer structure and, recognizing that the residence time is a function of the lipid molecule itself (i.e. its structure and the specific configuration that it is residing in), it is reasonable to expect that the residence time for
the overlayer lipid molecules to be significantly less than that for the those molecules that reside within a true bilayer (or micelle).

In the cases where overlayer dissipation occurred, the reattachment of intact bilayer islands during the reorganization phase was not observed, which indicates that the lipid molecules, once released from the monolayer, do not reassociate to form ordered solution species. These observations strongly suggest that reorganization of this transient monolayer involves reincorporation at the bilayer or defect edges. Furthermore, it is possible that phospholipids in the overlayer that became dislodged may reattach to the bilayer in the same orientation and in the same, or different, area. Over longer periods of time, however, these free molecules in solution were reincorporated into the existing bilayer, and there was no evidence of overlayer regrowth. Under high salt conditions, the direct absorption and unfolding of intact vesicles was not observed. Rather, the expansion of the monomolecular overlayer was detected. This would suggest that the soluble lipid molecules self-associate directly with the bilayer and the monolayer at the periphery of the growing overlayer.

These observations may be qualitatively accounted for in the following manner. These overlayers are transient structures that readily undergo reorganization on the time scale of minutes. Such overlayer structures have never been previously reported in other scanning probe microscopy studies of lipid bilayers. This may be attributed to the fact that most bilayer studies are performed on samples prepared by LB techniques, and which therefore, are typically in a gel or semi-crystalline state. The overlayer may be a consequence of the less perturbative free fusion approach adopted in the present study. Prior bilayer studies have typically employed contact mode imaging, an approach known
to impart significant lateral shear forces on the sample surface. While it is clear that contact mode scanning probe microscopy can reliably image bilayer and bilayer fusion, it is also well understood that the contact forces and surface pressures can in fact lead to reorganization of the bilayer and scanning artifact.\textsuperscript{12,24-26} In this study, tapping mode AFM was employed, which does not significantly distort or remove material.
3.6 CONCLUSION

By combining a flow-through system with the fluid cell of a scanning probe microscope, the ideal characteristics of supported lipid bilayers as biochemical substrates can be effectively exploited. This protocol affords the rare opportunity of examining not only the association of soluble ligands to these surfaces, but also nascent intrinsic dynamic bilayer assembly processes. This study has provided direct evidence of the dynamic in situ bilayer processes inherent to bilayer formation, including the presence of a heretofore unreported transient overlayer. This in situ approach provides an ideal means of probing the sequential assembly of single soluble ligands to model membrane substrates, observing dynamic events associated with in situ biomolecular complex formation, and studying the dynamics of bilayer formation and self-assembly.
3.7 REFERENCES


CHAPTER 4

Characterization of IMPDH-II at Supported Planar Bilayer Interfaces by Atomic Force Microscopy

4.1 OVERVIEW

Atomic force microscopy (AFM) is an effective high-resolution imaging technique, capable of providing detailed topographical information about biomolecular structures adsorbed at the solid-liquid interface. Not only is this technique suitable for examining the structure of individual molecules but the ability to perform such measurements in situ and in real-time suggests the possibility of observing dynamic biomolecular complex formation as it occurs under physiological conditions. Herein, human type II inosine 5’-monophosphate dehydrogenase (IMPDH-II) molecules adsorbed to differently charged supported planar bilayers (L-alpha-phosphatidylyserine, source bovine brain (PS), 1,2-dimyristoyl-phosphatidylcholine (DMPC), and 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP)) are characterized by in situ AFM. Comparison between the observed and theoretical structures of IMPDH-II revealed the presence of discrete structures identifiable as IMPDH-II monomers and tetramers. While these molecules adsorbed freely to neutral DMPC bilayers, they showed a marked preference for mica over the charged PS (negative) and DMTAP (positive) bilayers. This may be due to a chemical mismatch between these headgroups and the IMPDH-II molecules. Real time imaging of an IMPDH-II tetramer during complex formation with the inhibitor mycophenolic acid (MPA) was performed with no noticeable differences noted by section height analysis. This may, however, be due to limitations associated
with tip geometry, lack of information regarding specific orientation of the IMPDH-II tetramer, and a time scale proportional to structural changes.
4.2 MATERIALS

Computer Aided Chemistry for Education (CACHe)\textsuperscript{11} (Oxford Molecular Limited, Beaverton, OR, USA), Graphical Representations of Algorithms, Structures, and Processes (GRASP),\textsuperscript{12} and RasMol\textsuperscript{13} (Version 2.6; RasWin Molecular Graphics) modeling packages were used for all molecular modeling. CACHe (version 3.2) was used on a Power Macintosh 7300/200 outfitted with a XLR8 G300/200 G3 upgrade card under MacOS 8.6, and GRASP (version 1.3.6) was run on a Silicon Graphics R3000 Elan under IRIX 6.3. Lyophilized lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Purified IMPDH-II at 20 mg/ml in dialysis buffer (50 mM TRIS-HCL/8.0, 300 mM KCl, 10% Glycerol, 2 mM $\beta$Me, 2 mM EDTA) was provided by Mr. Steve Bryson and Mrs. Annie Cunningham (University of Toronto) and stored at −70 °C in 50 µl aliquots. Mica sheets were kindly provided by Dr. Eliot Chaikof at Emory University. Magnetic specimen preparation disks used with AFM were purchased from Ted Pella Incorporated (Redding, CA, USA). Silastic brand medical grade tubing (1/16” ID, 1/8” OD, Lot Number 72568) was purchased from Fisher Scientific Company (Pittsburgh, PA, USA). Oxide-sharpened silicon nitride V-shaped AFM cantilevers were purchased from Digital Instruments (Model DNPS – 120 µm; Santa Barbara, CA, USA). Water was distilled and deionized using Millipore Milli-Q UF Plus (Bedford, MA, USA) at 18 MΩ resistance. All other reagents were of the highest analytical grade.
4.3 METHODS

4.3.1 Molecular Modeling

Molecular models of IMPDH-II, and the lipid monolayers, PS, DMPC, DMTAP, were generated using RASMOL, GRASP, and CAChe. The IMPDH-II sequence used was published by Colby et al.\textsuperscript{14} and taken from the Protein Data Bank (1B3O). It is currently the only published structure of the human IMPDH-II form, and is based on a ternary complex containing the substrate analogue 6-chloropurine riboside 5'-monophosphate (6-CI-IMP) and the nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) analogue selenazole-4-carboxamide adenine dinucleotide (SAD). The approximate location of the active site binding pocket was determined by highlighting the substrate and NAD\textsuperscript{+} analogues. These models were used to estimate the approximate spatial dimensions of the IMPDH-II molecule. Molecular models of the IMPDH-II molecular and electrostatic surfaces were generated using the molecular modeling package GRASP. For the purposes of these models, the molecular surface is defined as the boundary of that volume within any probe sphere (meant to represent a water molecule with a probe radius of 1.4 Angstroms) of given radius sharing no volume with the hard sphere atoms that make up the molecule. To generate the surface electrostatic profile for the IMPDH-II complex, the Poisson-Boltzmann equation was solved within the GRASP package. Individual phospholipid molecules were built in CAChe based on known chemical structures and arranged in a $4 \times 2$ rectangular array. Energy minimization of this $4 \times 2$ phospholipid array generated the corresponding lipid monolayer models. The energy minimization routine locates a global energy minimum by measuring various energy terms (bond stretch, bond angle, dihedral angle, improper torsion, van der Waals, electrostatics, and
hydrogen bonds) associated with possible configurations of an initial structure. This process continues until the energy change between configurations is less than 0.001 KJ/mole, or until the molecule has been iterated 300 times. The models were used to develop conceptual models of the proposed interaction between IMPDH-II and the variously charged lipid bilayers.

### 4.3.2 Atomic Force Microscopy

Solution TappingMode™ atomic force microscopy (TMAFM) was performed on a Digital Instruments Nanoscope IIIA MultiMode scanning probe microscope (Digital Instruments; Santa Barbara, CA, USA) using 120 μm oxide-sharpened silicon nitride V-shaped cantilevers installed in a combination contact / TappingMode™ liquid flow cell. The cantilever tips were pre-treated for ~30 minutes with UV irradiation to remove adventitious organic contaminants. All TMAFM images were acquired using the E scanning head, which has a maximum lateral scan area of 14.6 μm x 14.6 μm, at tip scan rates from 1.50 – 2.50 Hz and cantilever drive frequencies of ~ 7-9 kHz. All images were captured as 512 x 512 pixel images and low-pass filtered. Feature size and volumes were calculated using the Digital Instruments Nanoscope software (version 4.21), and shareware image analysis program, NIH-Image (version 1.62).

### 4.3.3 IMPDH Addition on Lipid Bilayers

After obtaining stable images of the intact bilayers, imaging was halted and the tip withdrawn approximately 4 μm off the bilayer surface. Approximately 500 μl of 0.02 mg/ml IMPDH-II, in pH 8.0 PBS buffer, was then introduced into the AFM liquid cell
using a 27-gauge needle inserted into the Y–connector and allowed to sit for 30 minutes. The needle was removed and the tip re-engaged on the same spot prior to addition of IMPDH-II.

4.3.4 AFM In Situ IMPDH-II/MPA Complexation

High-resolution images of IMPDH-II molecules on lipid bilayers were acquired by in situ TMAFM. Imaging was halted and the tip withdrawn 4 μm. 1 mM inosine monophosphate (IMP) and 1 mM NAD$^+$ (in assay buffer (100 mM Tris/pH 8, 100 mM KCl, 3 mM EDTA)) were introduced into the AFM liquid cell using a 27-gauge needle inserted into the Y–connector. The needle was removed and the tip re-engaged immediately and imaging was continued for 30 minutes in order to capture any possible conformation changes upon substrate binding. Imaging was then halted and the tip withdrawn 4 μm. 100 mM MPA was introduced into the AFM liquid cell using a second 27-gauge needle inserted into the Y–connector. The tip was re-engaged immediately and imaging continued for 1 hour in order to capture any possible conformational changes upon inhibitor binding.
4.4 RESULTS

4.4.1 Molecular Modelling

4.4.1.1 IMPDH-II

Three-dimensional models generated in Rasmol portrayed the quaternary characteristics of the IMPDH-II tetramer, as well as the secondary and tertiary structural characteristics of the monomer, including the location of the active site binding pocket. As documented, native IMPDH-II exists as a tetramer with a crystallographic fourfold operation on the monomer. The shape of the tetramer can generally be thought of as a square, planar plate with bent corners. According to measurements on the crystalline structure, and assuming the disk is resting on its flat side, the tetramer had an approximate diagonal length of 13 nm, when measured from flanking domain to flanking domain (corner to corner), and height of 7 nm (Figures 4.4.1A and 4.4.1B).
Figure 4.4.1. Based on crystallographic studies, three-dimensional models of the IMPDH-II tetramer revealed that it is a square, plate-shaped molecule with bent corners. (A) The tetramer had a maximum diameter of approximately 13 nm when measure from tip of flanking domain to tip of flanking domain. (B) The tetramer had a maximum height of approximately 7 nm.
When examining the structure of the monomer, the α/β barrel making up the core domain can also be thought of as a disk with the flanking domain protruding from one end of the core domain as a small disordered spherical-shaped region. The entire monomer may be described as flat pear-shaped (Figure 4.4.2) with a length of \(~ 6 \text{ nm}\) and a diameter of \(~ 5 \text{ nm}\) (Figure 4.4.2A). When considering the monomer lying on its side, the disk of the core domain had an approximate height of \(~ 4 \text{ nm}\) and the flanking domain had an approximate height of \(~ 3.5 \text{ nm}\) (Figure 4.4.2B).

![Figure 4.4.2. Based on crystallographic studies, three-dimensional models of the IMPDH-II monomer revealed that it is pear-shaped. (A) The maximum length of the monomer, measured from flanking domain to core domain, was approximately 6 nm. The maximum diameter of the core domain was 5 nm. (B) The monomer had a core domain height of approximately 4 and a flanking domain height of approximately 3.5 nm.](image-url)
The electrostatic profile of the IMPDH-II tetramer had a distinct charge separation with a very large positive electrostatic region on one side of the disk and a smaller negative electrostatic region on the other side (Figure 4.4.3A). Due to the fourfold symmetry of the molecule, each monomer had a similar charge distribution. The active site binding pocket resides in the negative region, directly opposite the large positive region (Figure 4.4.3B).

Figure 4.4.3. Electrostatic profiles ($\pm 1kT$) mapped to the calculated molecular surface of IMPDH-II. (A) and (B) The tetramer has a distinct charge separation with a large positive region (blue) directly opposite a large negative region (red). (C) and (D) The monomer also has a similar charge distribution. As indicated by the yellow circle in (D), the active site binding pocket lay in the negative region, directly opposite the large positive region.
4.4.1.2 PHOSPHOLIPID MONOLAYERS

Individual phospholipid molecules were constructed in CAChE and monolayer sheets of $4 \times 2$ phospholipid molecules were created by energy minimized using CAChE. Three-dimensional structural models of the three phospholipid monolayers are shown (Figures 4.4.4A, 4.4.4B, and 4.4.4C).

Figure 4.4.4. Three-dimensional models of phospholipid monolayers consisting of 8 phospholipid molecules. (A) PS, (B) DMPC, and (C) DMTAP monolayers.
Taking into consideration the distinct charge separation in the electrostatic profile of IMPDH-II, the protein should associate itself with charged surfaces in a specific manner. With a negatively or positively charged bilayer surface (PS or DMTAP), IMPDH-II molecules should associate themselves with the oppositely charged electrostatic region closest to the charged phospholipid head groups (Figures 4.4.5A and 4.4.5C). With a neutral bilayer (DMPC), IMPDH-II molecules should orient themselves in a random fashion on the lipid bilayer surface (Figure 4.4.5B). The location of the active site binding pocket is noted in each case.
Figure 4.4.5. Theoretical orientations of IMPDH-II monomers resting on (A) negative PS monolayers (surface charge shown in red); (B) neutral DMPC monolayers; and (C) positive DMTAP monolayers (surface charge shown in blue). The position of the active site is shown in (A) and (C) with a yellow circle.
4.4.2 AFM Analysis of IMPDH-II on Bilayer Surfaces

4.4.2.1 L-ALPHA-PHOSPHATIDYLSERINE

*In situ* AFM imaging on the negatively charged PS bilayers was performed approximately 30 minutes after addition of 0.02 mg/ml IMPDH-II to the imaging environment. Initial AFM height images revealed a small population of adsorbed globular molecules on the bilayer surface; approximately 2 – 3 molecules / 2 μm². High-resolution height images revealed the recurring presence of circular shaped molecules (Figure 4.4.6A) among the various shapes. These molecules also showed a notable phase contrast from the underlying bilayer (Figure 4.4.6B). The observed uncorrected feature dimensions (height and diameter) were measured by section height analysis (Figure 4.4.6C). Appendix IA accounts for the range of possible over-estimation for each measurement due to the geometry of the tip. AFM measurements of height and diameter, as well as actual measurements and range of over-estimation due to tip geometry are presented in Table 4.4.1. Reported measurements were based on five sets of experimental results.
Figure 4.4.6. *In situ* tapping mode AFM (A) height and (B) phase images acquired in pH 8 PBS buffer of a circular-shaped IMPDH-II molecule on a PS bilayer. (C) Section analysis on high-resolution images of these structures revealed apparent height and diameter measurements (values reported in Table 1). Image sizes: 500 nm x 500 nm. Scan rate: 2.26 Hz. Scale bar: 100 nm.
The IMPDH-II molecules exhibited preference to the exposed regions of mica rather than the PS bilayer surface. After adding 0.02 mg/ml IMPDH-II to the system, holes in the bilayer began to fill in. The filling of the bilayer holes was clearly detected in both height and phase imaging (Figures 4.4.7A and 4.4.7B).

Table 4.4.1. AFM measurements, actual measurements, and range of over-estimation for the height and diameter of circular-shaped IMPDH-II molecules on PS bilayers. Each value in the “AFM Measurement” section is the mean ± S.D. of five determinations. * Refer to Appendix IA for calculations.
Figure 4.4.7. (A) *In situ* tapping mode AFM height image acquired in pH 8 PBS buffer of discontinuous PS bilayers after the addition of IMPDH-II to the imaging environment. (B) AFM phase image of (A) showing phase contrast between the existing PS bilayer and the region of bilayer holes filled with IMPDH-II molecules. Image sizes: 10 μm x 10 μm. Scan rate: 1.97 Hz. Scale bar: 1 μm.
4.4.2.2 1,2-Dimyristoyl-Phosphatidylcholine

*In situ* AFM imaging on neutral DMPC bilayers was performed approximately 30 minutes after addition of 0.02 mg/ml IMPDH-II to the imaging environment. At concentrations above 0.02 mg/ml, plaque-like protein structures would form on the bilayer surface. Imaging revealed the presence of adsorbed globular molecules on the bilayer surface with varying shape and size; approximately 10 molecules/2 μm². High-resolution imaging revealed a common structural motif of circular-shaped molecules (Figure 4.4.8A). All molecules also showed clear phase contrast relative to the underlying bilayer (Figure 4.4.8B). Section analysis on these circular structures (Figure 4.4.8C) provided height and diameter values. AFM height (Figure 4.4.9A) and phase (Figure 4.4.9B) images also revealed the steady appearance of pear-shaped molecules; approximately 20 molecules/2 μm². Consistent with resting on a neutral substrate, these structures were seen in different orientations (Figure 4.4.9C). Section analyses were performed on the pear-shaped molecules to determine the maximum length, and height of the base (the bulbous end of the structure), and neck of the structure (Figure 4.4.9D). The measurements derived from AFM are shown in Table 4.4.2. Reported measurements were based on five sets of experimental results. Furthermore, on DMPC surfaces the ratio of pear-shaped molecules relative to circular-shaped molecules was approximately 2:1.
Figure 4.4.8. *In situ* tapping mode AFM images acquired in pH 8 PBS buffer on DMPC bilayers after the addition of IMPDH-II. (A) AFM height imaging revealed the presence of various globular molecules with the consistent presence of circular-shaped molecules (marked by the arrows). (B) All adsorbed molecules had clear phase contrast. (C) Section height analysis was performed on these structures to determine height and diameter (values reported in Table 2). Image sizes: 1 μm x 1 μm. Scan rate: 2.00 Hz. Scale bar: 200 nm.
Figure 4.4.9. *In situ* tapping mode AFM images acquired in pH 8 PBS buffer on DMPC bilayers after the addition of IMPDH-II. Along with circular-shaped molecules, height (A) and phase (B) imaging also revealed pear-shaped molecules. (C) Consistent with resting on a neutral surface, the pear-shaped molecules oriented themselves in a random fashion. (D) Section height analysis was performed on these structures to determine height and diameter (values reported in Table 2). Image sizes: (A) and (B): 200 nm x 200 nm; (C): 600 nm. Scan rate: 2.00 Hz. Scale bar: (A) and (B): 40 nm; (C): 120 nm.
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<th>*Range of Over-Estimation</th>
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Table 4.4.2. AFM measurements, actual measurements, and range of over-estimation for height and diameter measurements of circular-shaped and pear-shaped IMPDH-II molecules on DMPC bilayers. Each value in the “AFM Measurement” section is the mean ± S.D. of five determinations. *Refer to Appendix IB for calculations.

4.4.2.3 1,2-DIMYRISTOYL-3-TRIMETHYLLAMMONIUM-PROPANE

*In situ* AFM imaging on positively charged DMTAP bilayers was performed approximately 30 minutes after addition of 0.02 mg/ml IMPDH-II to the imaging environment. Initial AFM height images indicated the small population of adsorbed globular molecules on the bilayer surface; approximately 2 – 3 molecules / μm². Consistent with the PS and DMPC cases, high-resolution AFM images of these molecules revealed a common circular-shaped motif (Figure 4.4.10A), and exhibited striking phase contrast from the underlying bilayer (Figure 4.4.10B). Section analysis performed on high-resolution images of these structures (Figure 4.4.10C) revealed height
and diameter measurements. These values and the range of over-estimation (Refer to Appendix IC) are reported in Table 4.4.3. Reported measurements were based on five sets of experimental results.

Figure 4.4.10. In situ tapping mode AFM height (A) and phase (B) images acquired in pH 8 PBS buffer of a circular-shaped IMPDH-II molecule on a DMTAP bilayer. (C) Section analysis on high-resolution images of these structures revealed apparent height and diameter measurements (values reported in Table 3). Image sizes: 500 nm X 500 nm. Scan rate: 2.26 Hz. Scale bar: 100 nm.
Table 4.4.3. AFM measurements, actual measurements, and range of over-estimation for height and diameter measurements of circular-shaped and pear-shaped IMPDH-II molecules on DMTAP bilayers. Each value in the “AFM Measurement” section is the mean ± S.D. of five determinations. * Refer to Appendix IC for calculations.

As seen with PS bilayers, it was also observed that at 0.02 mg/ml, IMPDH-II molecules preferred to adsorb to exposed regions of mica rather than the DMTAP bilayer surface. After adding IMPDH-II to the system, holes in the bilayer began to fill in. The filling of the bilayer holes was clearly detected in height and phase imaging (Figures 4.4.11A and 4.4.11B).
Figure 4.4.11. (A) *In situ* tapping mode AFM height image acquired in pH 8 PBS buffer of discontinuous DMTAP bilayers after the addition of IMPDH-II to the imaging environment. (B) AFM phase image of (A) showing phase contrast between the existing DMTAP bilayer and the region of bilayer holes filled with IMPDH-II molecules. Image sizes: 2 μm x 2 μm. Scan rate: 1.97 Hz. Scale bar: 400 nm.
4.4.3 AFM In Situ IMPDH-II/MPA Complexation Using PS Bilayers

*In situ* IMPDH-II/MPA complexation on PS bilayers was performed while imaging via AFM by capturing images after addition of agents involved in the complexation. An initial image of an IMPDH-II molecule was captured prior to addition of IMP and NAD\(^+\) to the imaging environment (Figure 4.4.12A). Figure 4.4.12B shows the IMPDH-II molecule 7 minutes after the addition of the substrate and cofactor. Figure 4.4.12C shows the molecule 4 minutes after the addition of MPA to the system. Figure 4.4.12D shows IMPDH-II 3 minutes after exchanging the imaging buffer back to pH 8 PBS in order to possibly visualize MPA release from the complexed enzyme. Section height analysis revealed no noticeable change in the IMPDH-II molecule during the complexation or release of the inhibitor.
Figure 4.4.12. *In situ* IMPDH-II/MPA complexation on PS bilayers visualized by AFM. (A) IMPDH-II molecule before addition of substrate and cofactor. (B) IMPDH-II molecule 7 minutes after addition of substrate and cofactor. (C) IMPDH-II molecule 4 minutes after addition of inhibitor, MPA. (D) IMPDH-II molecule 3 minutes after exchanging imaging buffer with pH 8 PBS. Image sizes: 200 nm x 200 nm. Scan rate: 2.54 Hz. Scale bar: 40 nm.
4.5 DISCUSSION

Capable of providing direct high-resolution images, AFM is exceptionally suited for providing detailed topographical information about biomolecular structures adsorbed at the solid-liquid interface. This technique is effective for molecular-level examination of proteins. The orientation properties of immunoglobulins and specific molecular conformations of plasmid DNA anti-Z-DNA antibodies have been studied by AFM. Supported planar membranes (SPBs) are effective substrates for surface-specific techniques such as AFM. Further, owing to the flexibility in tailoring the surface charge of SPBs, detailed information regarding orientation and spatial distribution of adsorbed molecules is also possible. This can be applied to preferentially orienting enzymes in order to observe ligand binding. Dynamic events such as proteolysis of collagen molecules, interactions between DNA condensates, and binding of protein to mRNA have been previously studied. Thus, not only is AFM suitable for examining the structure of individual molecules, but the ability to perform such measurements in situ and in real-time also gives rise to the possibility of observing dynamic biomolecular complex formation as it occurs under physiological conditions.

4.5.1 AFM Analysis of IMPDH-II on Bilayer Surfaces

After obtaining images of formed SPB surfaces, protein dilution buffer (pH 8 PBS) was flushed into the liquid cell and allowed to sit for ~30 minutes. Subsequent AFM imaging revealed no change in the SPB surfaces and this was used as a control. AFM imaging of these surfaces after the addition of 0.02 mg/ml IMPDH-II in dilution buffer revealed circular-shaped molecules on all three SPB surfaces. These molecules
were more prevalent on the neutral (DMPC) surfaces than on the charged surfaces (PS, DMTAP). On the neutral surfaces other globular species with varying shape and size were also found. Concurrent phase imaging revealed marked phase shift associated with all the adsorbed molecules. This is a clear indication that these molecules possessed different mechanical and/or electrostatic properties than the underlying bilayer or mica surfaces. A close correspondence between the molecular models of the IMPDH-II tetramer and the circular-shaped structures observed by AFM (Figure 4.5.1) was also noted. Differences in specific orientations of these molecules, however, could not be determined.

Figure 4.5.1. Three-dimensional AFM height image of a circular-shaped molecule adsorbed to DMPC bilayers. Correlation to a three-dimensional representation of an IMPDH-II tetramer reveals a resemblance of the tetramer resting on a bilayer with the flanking domains embedded into the surface. Image size: 500 nm x 500 nm. Scan rate: 2.26 Hz.
Also observed on DMPC bilayers was the presence of pear-shaped molecules. These molecules had a consistent pear shape and were found in different orientations. Concurrent phase imaging revealed that these molecules showed a slight phase shift compared to the bilayer and mica surfaces. Again, when comparing the three-dimensional structure of the IMPDH-II monomer to these pear-shaped molecules, there was a strong correlation between the two shapes (Figure 4.5.2). Further, consistent with resting on a neutral substrate, these molecules were found in different orientations (Figure 4.5.3) consistent with possible orientations of the IMPDH-II monomer.

Figure 4.5.2. AFM height image of a pear-shaped molecule adsorbed to a DMPC bilayer. Comparison with a three-dimensional model of an IMPDH-II monomer shows a strong correlation to the observed structure. Image size: 100 nm x 100 nm. Scan rate: 2.26 Hz.
Figure 4.5.3. AFM height image of pear-shaped molecules adsorbed to DMPC bilayers in various orientations. These molecules show a strong correlation to IMPDH-II monomers in random orientations.

Tables 4.4.1, 4.4.2, and 4.4.3 (refer to Results section) list the lateral and vertical measurements on the circular molecules on negative, neutral, and positive surfaces respectively. In each case, the height measurements were smaller and the diameters were larger compared to the actual dimensions. It should be noted here that the known three-dimensional structure of IMPDH-II is based on the conformation the protein assumes in
the crystalline state, which would be very ordered and rigid. In the soluble state, however, the conformation would be less well-defined. When examining the three-dimensional structure of the IMPDH-II tetramer (Figure 4.5.4A and 4.5.4C), it can be described as square planar disk with the corners angled. In solution, however, the contacts between adjacent subunits would be more relaxed and the structure may vary. Also, due to inter-species variation, the residues compromising the region that connects the core domain to the flanking domain have been described as a flexible hinge. For these reasons the crystalline bowl-like structure may relax in the soluble state and assume a more planar shape (Figure 4.5.4B and 4.5.4D). This flattening of the tetramer would not only decrease the measured height, but also cause an increase in the lateral dimensions.

Figure 4.5.4. Side view of the IMPDH-II tetramer. (A) and (C) The crystallographic structure has a bowl-like structure. (B) and (D) In solution, however, the protein may flatten into a more planar-like structure.
Further, due to the flexible nature of SPB surfaces, IMPDH-II tetrmers may nestle into the phospholipid head groups (Figure 4.5.5). This would particularly apply to the flanking domain region, as it is considerably smaller than the core domain region of the tetramer and could settle into the bilayer to a greater degree. Together, these factors would contribute to a measured height that was less than expected for the IMPDH-II tetramer. AFM height measurements, however, could not provide conclusive evidence of the orientation of the circular-shaped molecules should they be IMPDH-II tetrmers. The diameter measurements of the circular molecules were also larger compared to measurements performed on the three-dimensional IMPDH-II structure. Here, the finite dimensions of the tip are an important consideration. Adapting an equation that calculates the over-estimation involved in imaging spheres, calculations involving the radius of curvature of the tip compared with the size of the structure under scrutiny provided an acceptable range of over-estimation for the IMPDH-II tetramer (Appendices IA – IC). For the circular molecules it was found that the diameter determined by AFM was in the acceptable range of over-estimation for each SPB substrate.
Figure 4.5.5. IMPDH-II tetramers in different orientations (A) and (B) showing how the structure may nestle into a phospholipid bilayer. Figure not drawn to scale.

Table 4.4.2 (refer to Results section) lists the vertical and lateral measurements of the pear-shaped molecules on neutral bilayers. As with the circular molecules, the height measurements were smaller than expected and the length measurements were larger than expected. The argument that the IMPDH-II monomer is embedding into the bilayer can also be applied here. This would result in a smaller measured height (Figure 4.5.6).
Further, the measured lengths of these molecules were within the possible range of over-estimation when taking the dimensions of the tip into account (Appendix IB).

Figure 4.5.6. Schematic diagram of how the IMPDH-II monomer may nestle into a phospholipid bilayer. Figure not drawn to scale.

As a result, the circular- and pear-shaped molecules showed several crucial characteristics: they were not present in the control dilution buffer; they possessed increased phase shift compared to SPBs and mica indicating they had different mechanical and/or electrostatic properties; they had similar shapes compared to three-dimensional models of IMPDH-II tetramers and monomers; and they showed vertical and lateral dimensions that were within an acceptable range of over-estimation for AFM measurements of IMPDH-II tetramers and monomers. Thus, it is likely that these molecules were IMPDH-II tetramers and monomers, respectively. Furthermore, the approximate ratio of 2:1 for IMPDH-II monomers relative to tetramers at 0.02 mg/ml supports the observation by non-denaturing PAGE where the monomer is the predominant structure at this concentration.
It should also be noted that the lateral measurements of the IMPDH-II tetrabers resting on charged bilayers were larger than on neutral bilayers. This may be due to the charge present on the SPB surfaces interacting with the nominal negative charge on the silicon nitride imaging tip. The surface chemistry of tips and effects of tip-sample interactions have been well documented.\textsuperscript{24,25} When imaging negative surfaces, such as the PS bilayers, the repulsive interactions between the SPB and the tip would result in a loss of resolution and contrast.\textsuperscript{26} It has also been shown that imaging surfaces using an ‘attractive’ interaction regime between tip and sample results in enhanced resolution.\textsuperscript{26} In this study, however, the positive charge associated with the DMTAP surface proved to be too great since extended imaging frequently resulted in the tip being drawn into perturbative contact with the surface. In order to counteract both of these charge interactions for PS and DMTAP, the distance of the tip above the surface was increased, which resulted in reduced resolution and contrast. This would result in more convoluted and over-estimated lateral measurements.

While the biochemical characterization of IMPDH-II showed the existence of an equilibrium between IMPDH-II tetrabers, dimers, and monomers at 0.02 mg/ml, AFM performed on IMPDH-II at this concentration on SPB interfaces revealed the presence of tetrabers and monomers only. Examination of IMPDH-II on neutral surfaces revealed the presence of various globular shaped species, some of which did not have a defined or constant shape (refer to Results section; Figure 4.4.8). There were two shapes, however, that were consistently identified. These were the circular- and pear-shaped IMPDH-II molecules. All other shaped molecules could not be assigned a definite representative shape. It is possible that these molecules may have been IMPDH-II dimers, trimers, and
aggregates of various sizes. It is also important to note that phospholipid bilayers provide a biomimetic substrate suitable for protein adsorption and, owing to its fluid and dynamic nature, conformational and aggregation processes. Extended duration imaging of IMPDH-II molecules indicated, however, that aggregation of monomers to dimers or tetramers was not taking place. Since IMPDH-II molecules did not reassociate on fluid bilayer surfaces, our contention made in Chapter 2 that IMPDH-II molecules do not reassociate on PVDF membrane surfaces, which bind protein molecules with strong hydrophobic interactions, was confirmed.

In the presence of the charged PS and DMTAP lipid bilayers, we noted that the IMPDH-II molecules preferentially adsorbed to the open mica surfaces, and rarely to the surface of the lipid bilayers. We suggest that the behaviour is not driven by specific electrostatic stabilization of the IMPDH-II molecules at the lipid surface but rather by a chemical mismatch between the PS or DMTAP headgroups and the IMPDH-II molecules. Under these circumstances, the IMPDH-II molecules will preferentially bind to the exposed mica surface. We note that specific association of soluble molecules with lipid headgroups has been demonstrated for the interaction of the amyloid-β peptide.²⁷

### 4.5.2 AFM In Situ IMPDH-II/MPA Complexation Using PS Bilayers

After obtaining images of IMPDH-II tetramers on PS bilayers, imaging was halted and reagents (IMP, NAD⁺, and MPA) involved in the IMPDH-II/MPA complexation were added. Imaging was re-initiated after each step and continued for several minutes prior to the next step. Dilution buffer was then added in a final step in order to view release of the inhibitor. As encountered previously, the charge associated
with the PS bilayer affected the resolution during imaging. As a result, no noticeable differences were noted by section height analysis after each step. The geometry of the tip may have been a factor, however, since the IMPDH-II binding pocket lies at the interface between adjacent monomers, which may have been inaccessible by the tip. Also, since the specific orientation of the IMPDH-II tetramer was unknown, the actual location of each IMPDH-II active site could not be discerned for section height analysis. Finally, the IMPDH-II catalyzed reaction proceeds very quickly (~ 1.3 turnovers/molecule/sec), thus limitations associated with AFM scan-intervals proportional to the rate of structural change may have also posed a problem.
4.6 CONCLUSION

AFM is well suited for providing detailed topographical information about biomolecular structures adsorbed at the solid-liquid interface. By using supported planar bilayers as substrates for protein adsorption, we have characterized IMPDH-II molecules at the protein-lipid interface by AFM. Adsorption of 0.02 mg/ml IMPDH-II on charged surfaces revealed the presence of circular–shaped molecules. Structural studies by *in situ* atomic force microscopy on neutral bilayers revealed the presence of various globular–shaped species with the consistent presence of circular–shaped molecules, along with pear–shaped molecules. Following careful scrutiny, it is likely that these structures were IMPDH-II tetramers and monomers, respectively. Another important factor to consider when using AFM is the nature of the substrate. Due to a chemical mismatch between PS and DMTAP headgroups, IMPDH-II displayed a marked preference for mica over these surfaces over extended periods of time.
4.7 REFERENCES


11. CAChe WorkSystem, Version 3.9, CAChe Group, Fujitsu Systems Business of America, Inc


CHAPTER 5

Summary

5.1 CONCLUSIONS

The results obtained from these studies clearly demonstrate that human type II inosine 5'-monophosphate dehydrogenase (IMPDH-II) exists as a reversible, self-associating system at concentrations relevant for kinetic studies and \textit{in vivo}. Biochemical and structural analysis of IMPDH-II at varying concentrations has revealed that at low protein concentrations, IMPDH-II exists as a mixture of monomers, dimers, and tetramers. As protein concentration increases, however, the tetrameric species dominates. Structural studies by \textit{in situ} atomic force microscopy confirmed the existence of IMPDH-II monomers and tetramers at similar concentrations. Furthermore, the isolation of monomeric IMPDH-II structures lead to the interpretation that this structure retains activity. The combination of these powerful techniques has created a unique protocol for the biophysical study of IMPDH-II and has created significant new insights into its association characteristics and activity.

5.2 FUTURE RECOMMENDATIONS

The information obtained by this research project has provided significant insights into the self-association characteristics of IMPDH-II and establishes a solid foundation for more in-depth studies of the association–dissociation mechanisms of IMPDH-II by techniques such as analytical ultracentrifugation and florescence studies. The analytical ultracentrifuge combines a high-speed centrifuge with an optical system
and enables one to acquire the position of sedimenting boundaries as a function of time.\textsuperscript{1} In the case of self-associating systems, one may obtain thermodynamic information about the macromolecule. This includes molecular mass averages as a function of concentration, which enables one to determine stoichiometries and equilibrium constants of the interacting species.

At physiological ionic strength most protein aggregates do not show appreciable dissociation when observed at the lowest concentrations (> $10^{-6}$ M) at which the sedimentation properties can be reliably determined in the ultracentrifuge.\textsuperscript{1} Fluorescence techniques, however, are an appropriate application to the study of the reversible association of proteins, as it is sensitive to solutions of fluorophores at concentrations at $10^{-6}$ to $10^{11}$ M.\textsuperscript{1} Concentration–dependent changes in spectral distribution, quantum field, lifetime, or polarization can be used to follow the dissociation characteristics of the protein.\textsuperscript{1} Polarization observations either from the intrinsic fluorophores of the protein, such as tyrosine and tryptophan residues, or from covalently attached fluorescent labels depend directly on the rate of rotation of the fluorophore and thus contain information about the dynamics of the fluorescent unit. While fluorescence–based techniques are highly sensitive, native IMPDH-II does not contain any tryptophan residues and thus would require appropriate labelling.

This study has demonstrated the effectiveness of atomic force microscopy for providing detailed topographical information about biomolecular structures adsorbed at the solid-liquid interface. This technique also provides an effective complement to spectroscopic and diffraction-based approaches for studying crystalline structures. These observations include \textit{in situ} determination of crystal packing,\textsuperscript{2} space group symmetry,\textsuperscript{2}
defect formation, visualization of crystal growth mechanisms, and direct identification of crystal polymorphs. While X-ray diffraction can provide high-resolution atomic scale data, it samples the bulk structure of the crystal. AFM, as a surface technique, supplements such data by providing direct characterization of local interfacial structure, specifically, on actively growing, low energy, crystal faces, as recently demonstrated for insulin. The direct observation of the dynamic processes associated with IMPDH-II crystal formation will provide direct determination of molecular packing motifs, as well as precise molecular scale details of the crystal nucleation, growth, and defect generation mechanisms associated with the formation of three-dimensional protein crystals. Thus, AFM is ideally suited to determine the optimal conditions for crystal growth. This is particularly important since crystal structures of ligand-free IMPDH-II have not been of suitable quality for diffraction-based analysis. By directly observing the effect of crystallization conditions on crystal face growth rates, mechanisms of growth, and propensity for dislocation formation, it will be possible to determine optimal conditions for growth of ligand-free IMPDH-II crystals. AFM can also be used to observe the dynamic processes associated with human IMPDH-II/MPA complex crystal formation. This is also relevant since the crystal structure of this enzyme-inhibitor complex has not been resolved. Such data may have more detailed implications in terms of the identification of specific intermolecular contacts and binding domain interactions, and molecular packing motifs for the IMPDH-II/MPA complex.

Furthermore, this study has demonstrated that the monomeric form of IMPDH-II can be assigned catalytic activity. This may have an impact on the design of specific IMPDH-II inhibitors, thus a thorough investigation into the relationships between the
different IMPDH-II structures and their activity would be valuable. This study utilized the reversible, self-associating behaviour of IMPDH-II in order to study monomer activity; however, for more accurate investigations, IMPDH-II monomers and dimers must be produced and stabilized to prevent reassociation. Generating specific mutations in the IMPDH-II sequence may be a useful technique for achieving this goal. Close inspection of the interface between IMPDH-II monomers revealed that critical short range (> 3.0 Å) hydrogen bonds form between the backbone at residue positions Ile-37, Gly-40, and Ile-42 and various residues from adjacent subunits, thus stabilizing the tetrameric structure. Computer modelling verified that mutating these residues to Trp-37, Pro-40, and Tyr-42 results in significant steric hindrances due to the larger size of the new amino acid side chains. Introducing unbalanced charges in hydrophobic patches of monomer-monomer contacts could be another way to destabilize the oligomers. Carefully designed mutations may even provide the proper balance between interference and stabilization of subunit interactions that could result in IMPDH-II dimers. Sufficiently stabilized in such ways, the relative kinetic properties of these structures could then be properly investigated.
5.3 REFERENCES


APPENDICES

Appendix I
Calculation of over-estimation in lateral AFM measurements

In a study of the assembly structures of Tobacco Mosaic Virus (TMV) an equation was developed to approximate the amount of lateral over-estimation due to the geometry of the AFM imaging tip. From x-ray diffraction measurements, the TMV structures were known to be rod-shaped, thus a transverse section of these particles must be a circle. Due to the shape of the tip, however, section analysis on AFM images of these viruses were distorted by being wider toward the bottom (Figure 1). A theoretical schematic diagram of the interaction of the probe tip with the TMV particles was generated and used to confirm the amount of over-estimation.

![Diagram of interaction of probe tip with TMV particle](image)

Figure 1. The theoretical height profile (dark solid line) is illustrated together with a probe tip (with radius $R$) and the TMV particle (with radius $r$). In the resultant height profile, $h_{\text{max}}$ is the maximum height of the profile and $W_b$ is the width at the bottom of the profile respectively.
An equation was derived from this model that calculated $W_0$ from $R$ and $r$:

$$W_0 = 4\sqrt{Rr} \quad \text{for } \sin \theta < (R - r)/(R + r)$$

where $R$ is the radius of the tip apex ($5 - 40$ nm),

$r$ is the radius of the sample,

and $\theta$ is the cone angle of the tip ($2\theta = 35^\circ$).

This calculation can be applied to the section height analyses of the circular- and pear-shaped molecules observed in this study (Figure 2A and 2B).

Figure 2. Typical section height analysis profiles of (A) circular-shaped and (B) pear-shaped molecules.
By approximating a sphere that could fit into the ends of these two height profiles, the equation for $W_b$ can be applied to these measurements (Figure 3A and 3B).

Figure 3. By approximating spheres into the ends of the section height analysis for the (A) circular-shaped and (B) pear-shaped molecules, we can apply the equation for $W_b$ to our AFM measurements.
Taking into consideration that the radius of curvature of an AFM tip ranges from 5 to 40 nm, the equation will have minimum and maximum over-estimation values:

\[
W_s \min = \left( \frac{4\sqrt{5r_1}}{2} + \frac{4\sqrt{5r_2}}{2} \right)
\]

(1)

\[
W_s \max = \left( \frac{4\sqrt{40r_1}}{2} + \frac{4\sqrt{40r_2}}{2} \right)
\]

(2)

for \( \sin \theta < (R - r)/(R + r) \)

After the range is obtained, the actual length of the sample being measured should be added to each value to give an acceptable range of measurement.

It should be noted here that the study performed on the TMV particles used contact mode AFM and our study used TappingMode™ AFM. While the theoretical concept behind the over-estimation still applies, the measurements attained by TappingMode™ AFM may experience added convolution due to the intermittent contact with the surface.
Appendix 1A
Over-estimation of circular-shaped IMPDH-II molecules on PS bilayers.

$h_{\text{max}}_1 = h_{\text{max}}_2 = 4$ nm
Thus, $r_1 = r_2 = 2$ nm.

Substituting this value into Equation (1) yields a minimum over-estimation of:

$$W_b \min = \left( \frac{4\sqrt{5(2)}}{2} + \frac{4\sqrt{5(2)}}{2} \right)$$

$$W_b \min = 12.6 \text{ nm}$$

Substituting this value into Equation (2) yields a maximum over-estimation of:

$$W_b \max = \left( \frac{4\sqrt{40(2)}}{2} + \frac{4\sqrt{40(2)}}{2} \right)$$

$$W_b \max = 35.8 \text{ nm}$$

Test: $\sin{\theta} < (R - r)/(R + r)$ for $R = 5$ and 40 nm.

$$(5 - 2)/(5 + 2) > \sin(17.5) < (40 - 2)/(40 + 2)$$

$$0.43 > 0.30 < 0.90$$

The actual diameter determined using the crystallographic structure for an IMPDH-II tetramer is $\sim 13$ nm. Thus, the acceptable range of measurement is approximately 26 ($\sim 13 + \sim 13$) nm to 49 ($\sim 13 + \sim 36$) nm.
Appendix 1B
Over-estimation of circular-shaped IMPDH-II molecules on DMPC bilayers

\[ h_{\text{max}_1} = h_{\text{max}_2} = 3 \text{ nm} \]

Thus, \( r_1 = r_2 = 1.5 \text{ nm} \)

Substituting this value into Equation (1) yields a minimum over-estimation of:

\[ W_b^\text{min} = \left( \frac{4 \sqrt{5(1.5)}}{2} + \frac{4 \sqrt{5(1.5)}}{2} \right) \]

\[ W_b^\text{min} = 11.0 \text{ nm} \]

Substituting this value into Equation (2) yields a maximum over-estimation of:

\[ W_b^\text{max} = \left( \frac{4 \sqrt{40(1.5)}}{2} + \frac{4 \sqrt{40(1.5)}}{2} \right) \]

\[ W_b^\text{max} = 31.0 \text{ nm} \]

Test: \( \sin \theta < (R - r)/(R + r) \) for \( R = 5 \) and \( 40 \text{ nm} \).

\[ (5 - 1.5)/(5 + 1.5) > \sin(17.5) < (40 - 1.5)/(40 + 1.5) \]

\[ 0.54 > 0.30 < 0.93 \]

The actual diameter determined using a crystallographic structure for an IMPDH-II tetramer is \( \sim 13 \text{ nm} \). Thus, the acceptable range of measurement is approximately 24 (\( \sim 13 + \sim 11 \)) nm to 44 (\( \sim 13 + \sim 31 \)) nm.
Over-estimation of pear – shaped IMPDH-II molecules on DMPC bilayers.

\[ h_{\text{max}}_1 = 1.5 \text{ nm}, \text{ thus } r_1 = 0.75 \text{ nm} \]

\[ h_{\text{max}}_2 = 2 \text{ nm}, \text{ thus } r_2 = 1 \text{ nm} \]

Substituting this value into Equation (1) yields a minimum over-estimation of:

\[
W_b \text{ min} = \frac{4\sqrt{5(0.75)}}{2} + \frac{4\sqrt{5(1)}}{2}
\]

\[
W_b \text{ min} = 8.6 \text{ nm}
\]

Substituting this value into Equation (2) yields a maximum over-estimation of:

\[
W_b \text{ max} = \frac{4\sqrt{40(0.75)}}{2} + \frac{4\sqrt{40(1)}}{2}
\]

\[
W_b \text{ max} = 23.6 \text{ nm}
\]

Test: \(\sin \theta < (R - r)/(R + r)\) for \(R = 5\) and \(40\) nm.

For \(r_1\):

\[
\frac{(5 - 1.75)}{(5 + 1.75)} > \sin(17.5) < \frac{(40 - 1.75)}{(40 + 1.75)}
\]

\[0.48 > 0.30 < 0.92\]

For \(r_2\):

\[
\frac{(5 - 1.5)}{(5 + 1.5)} > \sin(17.5) < \frac{(40 - 1.5)}{(40 + 1.5)}
\]

\[0.54 > 0.30 < 0.93\]

The actual diameter determined using a crystallographic structure for an IMPDH-II monomer is \(\sim 6\) nm. Thus, the acceptable range of measurement is approximately \(15 (\sim 6 + \sim 9)\) nm to \(30 (\sim 6 + \sim 24)\) nm.
Appendix 1C
Over-estimation of circular–shaped IMPDH-II molecules on DMTAP bilayers

h_{\text{max}1} = h_{\text{max}2} = 4.5 \text{ nm}

Thus, r_1 = r_2 = 2.25 \text{ nm}

Substituting this value into Equation (1) yields a minimum over-estimation of:

\[ W_b \min = \left( \frac{4\sqrt{5(2.25)}}{2} + \frac{4\sqrt{5(2.25)}}{2} \right) \]

\[ W_b \min = 13.4 \text{ nm} \]

Substituting this value into Equation (2) yields a maximum over-estimation of:

\[ W_b \max = \left( \frac{4\sqrt{40(2.25)}}{2} + \frac{4\sqrt{40(2.25)}}{2} \right) \]

\[ W_b \max = 38.0 \text{ nm} \]

Test: \( \sin \theta < (R - r)/(R + r) \) for \( R = 5 \) and 40 nm.

\[ (5 - 2.25)/(5 + 2.25) > \sin(17.5) < (40 - 2.25)/(40 + 2.25) \]

\[ 0.38 > 0.30 < 0.89 \]

The actual diameter determined using a crystallographic structure for an IMPDH-II tetramer is \( \sim 13 \) nm. Thus, the acceptable range of measurement is approximately 26 (\( \sim 13 + \sim 13 \)) nm to 51 (\( \sim 13 + \sim 38 \)) nm.

REFERENCES

\footnote{Maeda, H. An atomic force microscopy study for the assembly structures of tobacco mosaic virus and their size evaluation. \textit{Langmuir} 1997, 13, 4150-4161.}