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Biophysical Characterization of NEMO and IKK2

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Chemistry

by

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2007
The dissertation of Devin Lee Drew is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2007
to my parents

I couldn’t have done it without you.
This is true of the many good people who are a part of my life.
    It is most true of my parents.

also to
    Gitte Vold
    and
    Murray Goodman
I am grateful for the brief yet significant ways that their lives touched mine.
EPIGRAPH

“Don’t back up any more than you need to.”

Bob Monahan
advice on driving, 1989
# TABLE OF CONTENTS

Signature Page ................................................................. iii
Dedication ........................................................................ iv
Epigraph ........................................................................... v
Table of Contents ............................................................ vi
List of Figures .................................................................... ix
List of Tables ....................................................................... xi
Abbreviations and Symbols ................................................ xii
Acknowledgments ............................................................. xv
Vita ....................................................................................... xvii
Abstract ............................................................................ xviii

1. Introduction ................................................................. 1
   1.1 NF-κB signaling ....................................................... 3
   1.2 The NF-κB and IκB proteins ..................................... 6
   1.3 The IκB kinase complex .......................................... 9
       1.3.1 The catalytic subunits: IKK1 and IKK2 .............. 9
       1.3.2 The NEMO subunit ........................................ 14
   1.4 Gene function in the NF-κB pathway ......................... 15
   1.5 IKK1, 2, and NEMO are required for canonical pathway signaling . 17
   1.6 NF-κB and disease ................................................ 18
   1.7 Structure in the NF-κB signaling paradigm ................. 19
   1.8 Focus of study ....................................................... 23

2. Purification and characterization of IKK2 ........................... 24
   2.1 Introduction ........................................................... 24
   2.2 Results ..................................................................... 25
       2.2.1 Baculovirus expression of IKK2 ...................... 26
       2.2.2 Identification of a stable N-terminal domain of IKK2 . 27
       2.2.3 Baculovirus expression of IKK2 1-667 .......... 27
       2.2.4 Expression of IKK2 1-664 in E. coli ............... 28
       2.2.5 Sedimentation experiments using analytical ultracentrifugation ................................................................. 31
       2.2.6 Dynamic light scattering .................................. 33
       2.2.7 Crystallization of His6Flag-IKK2EE 1-666 .......... 34
2.3 Discussion ................................................. 35

3. Purification and Characterization of Deletion Mutants of NEMO ........ 37
  3.1 Introduction ........................................... 37
  3.2 Results ................................................ 38
    3.2.1 Generation of NEMO N-terminal deletion mutants (NEMO1-130 and NEMO 40-90) ............ 38
    3.2.2 Generation of point mutants in NEMO 40-90 .......... 43
    3.2.3 Generation of other NEMO deletion mutants .......... 44
    3.2.4 Analysis of the secondary structure and foldedness of the NEMO N-terminal fragments .......... 46
    3.2.5 Sedimentation studies of NEMO 1-210 and NEMO 1-130 .. 48
  3.3 Discussion ............................................. 48

4. IKK2 Binding by NEMO ...................................... 54
  4.1 Introduction ........................................... 54
  4.2 Results ................................................ 54
    4.2.1 The N-terminal region of NEMO interacts with the catalytic IKK2 subunit .................. 54
    4.2.2 Oligomeric state of the IKK2 binding region of NEMO ..... 56
    4.2.3 Binding affinities of the NEMO and IKK2 complexes .... 58
    4.2.4 Purification of the IKK2/NEMO1-130 Complex .......... 60
  4.3 Discussion ............................................. 60

5. Discussion ................................................. 64

6. Materials and Methods ...................................... 69
  6.1 Molecular cloning and mutagenesis ............................ 72
    6.1.1 DNA purification .................................. 72
    6.1.2 PCR ................................................ 73
    6.1.3 Point mutations .................................... 74
    6.1.4 His6Flag-IKK2EE and His6Flag-IKK2EE1-666 ......... 74
    6.1.5 GST-IKK2EE1-664 ................................ 75
    6.1.6 GST-IKK2EE665-756 ................................ 76
    6.1.7 His6NEMO 1-130 .................................. 76
    6.1.8 His6NEMO 40-90 .................................. 76
    6.1.9 His6NEMO 1-210 and 40-210 .......................... 76
    6.1.10 His6NEMO 40-90 Mutants ............................ 77
  6.2 Protein over-expression and purification ....................... 77
    6.2.1 Verification of protein overexpression ................ 77
    6.2.2 Ni2+ chelation chromatography ....................... 78
    6.2.3 His6Flag-IKK2EE, and His6Flag-IKK2EE1-666 .......... 79
    6.2.4 GST-IKK2EE1-664 and GST-IKK2EE665-756 ............. 80
    6.2.5 His6-NEMO 1-130 .................................. 80
6.2.6 SR-IκBα .................................................. 81
6.2.7 cDNA sources ............................................. 81
6.2.8 His$_6$-IKK2$_{EE}$, His$_6$-NEMO 1-130 complex ............ 81
6.3 Crystallization .............................................. 82
6.4 Biophysical methods ...................................... 83
  6.4.1 Isothermal titration calorimetry ......................... 83
  6.4.2 Analytical ultracentrifugation .......................... 83
  6.4.3 Dynamic light scattering ............................... 84
  6.4.4 Circular dichroism spectrometry ...................... 85
  6.4.5 Fluorescence measurements .......................... 86
6.5 LCMS ....................................................... 87
  6.5.1 Analytical size exclusion chromatography ............... 88
6.6 Miscellaneous methods ................................... 89
  6.6.1 Microfuge tube cap dialysis ............................ 89
  6.6.2 Amino acid sequence alignment ......................... 89
A. Isothermal Titration Calorimetry Data ..................... 90
B. Preliminary CD spectra for NEMO 1-130 and NEMO 40-90 .... 93
C. Fluorescence results ....................................... 98
D. Establishment of a Cell Culture Lab for Exogenous Protein Expression with Baculovirus ......................... 100
E. Sequence Alignments ....................................... 104
  E.1 IKK Catalytic subunits aligned with PKA ................ 104
  E.2 Ubiquitin aligned with IKK2 and IKK1 .................. 106
F. cDNA Sources .............................................. 107
G. Expression of the Bovine NEMO Zinc Finger Domain ........ 109

Bibliography .................................................... 110
LIST OF FIGURES

1.1 κ light chain .......................... 1
1.2 NF-κB Signaling Overview .......................... 5
1.3 The IκB Destruction Box .......................... 6
1.4 NF-κB and IκB Proteins .......................... 7
1.5 The IKK Complex Proteins and Putative Domains .......................... 10
1.6 The Kinase Domain Activation Segment .......................... 12
1.7 NEMO Binding Region .......................... 14
1.8 NF-κB:DNA and IκB:NF-κB structures .......................... 21

2.1 AUC Measurements .......................... 25
2.2 His$_6$-IKK$_{2EE}$ peak fractions .......................... 26
2.3 Stable IKK2 fragment .......................... 27
2.4 IKK2 1-667 produced in insect cells .......................... 29
2.5 GST IKK$_{2EE}$ is expressed but not active from _E. coli_ .......................... 30
2.6 Nonideal behavior of IKK2 by AUC .......................... 32
2.7 Polydispersity and aggregation of IKK2 by DLS .......................... 33
2.8 Putative crystal of His$_6$Flag-IKK$_{2EE}$ 1-666 .......................... 35

3.1 Expression check for His$_6$-NEMO 1-130 .......................... 38
3.2 Purified NEMO 1-130 and 40-90 .......................... 39
3.3 Circular Polarized Light .......................... 41
3.4 Measurement of Ellipticity .......................... 41
3.5 Fit CD Deconvolution .......................... 42
3.6 His$_6$-NEMO 40-90 Mutants Purified .......................... 43
3.7 Expression and purification of NEMO deletion mutants .......................... 45
3.8 CD Spectra and thermal melts of NEMO 1-210, NEMO 40-210, and NEMO 1-130 .......................... 47
3.9 Sedimentation equilibrium data for NEMO 1-210, and NEMO 1-130 49
3.10 Assessment of NEMO fragment stability during AUC .......................... 50
3.11 Stable degradation product of NEMO 1-130 .......................... 51

4.1 N-terminus of NEMO interacts with the C-terminus of IKK2 .......................... 55
4.2 Determination of molecular weights of unbound and IKK2-bound NEMO using size exclusion chromatography .......................... 57
4.3 IKK2 and NEMO 1-130 AEX and Ni$^{2+}$ Purification .......................... 61
4.4 IKK2 and NEMO 1-130 complex by size exclusion .......................... 62

6.1 Protein Expression Constructs .......................... 71

A.1 ITC binding isotherm for NEMO 40-90 .......................... 90
A.2 ITC binding isotherms: NEMO 1-210 and 40-210 .......................... 91
A.3 ITC binding isotherms: NEMO 1-130 and 40-130 .......................... 92
# LIST OF TABLES

1.1 Gene Knockout Observations ............................................. 17
1.2 IKK, NF-κB Signaling and Disease ...................................... 20
4.1 ITC observations ............................................................... 58
6.1 Cloning with Complementary Overhangs .............................. 75
6.2 Primers for point mutations ................................................. 77
D.1 Cell culture facility starting cost estimates .......................... 101
ABBREVIATIONS AND SYMBOLS

AEX  Anion exchange

APS  Ammonium Persulfate

ATP  Adenosine triphoshatate

CTC  IKKγ C-terminal coiled-coil region, human aa 258–358

DD  Dimerization Domain

DTT  Dithiothreitol

EDTA  Ethylene Diamine Tetra-Acetic acid

EE  Indicates dual Ser to Glu mutations in the kinase domain activation segment, eg IKK2EE

GST  Glutathione S-transferase

His6  A protein fusion tag consisting of 6 sequential His residues

HIV  Human Immunodeficiency Virus

HSV  Herpes Simplex Virus

IκB  Inhibitor of NF-κB

IKK  IκB Kinase

IKK1  IκB Kinase-1 [45], also known as IKKα [44].

IKK2  IκB Kinase-2 [45], also known as IKKβ [50].

IKKγ  IκB Kinase γ subunit [57], synonymous with NEMO [60], Fip-3 [71], and IKKAP1 [73]
IL-1  The proinflammatory cytokine Interleukin-1

IL-1R  Interleukin-1 Receptor

IP  Incontinentia Pigmenti, a X-linked human disorder

IP, co-IP  Immunoprecipitate, co-immunoprecipitate

IPTG  isopropyl-β-d-thiogalactopyranoside

kDa  Kilodalton, $10^3 g / N_a$

LCMS  Mass Spectrometry preceded by Liquid Chromatography

LPS  Lipopolysaccharide, also known historically as Endotoxin.

LZ  Leucine zipper [15]

MOI  Multiplicity of Infection

NaCl  Sodium Chloride (aka Table Salt)

NBD  NEMO Binding Domain. Name given to a six amino acid identity among
      the C-termini of IKK1 and IKK2 [79].

NBR  NEMO Binding Region, the author’s preferred description of the NBD.¹

NEMO  NF-κB essential modulator [60],
      synonymous with IκB Kinase γ [57],
      Fip-3 [71], and IKKAP1 [73]

NF-κB  Nuclear Factor κ B

NTC  IKKγ N-terminal coiled-coil region, human aa 93-197

NTD  N-terminal Domain

¹NEMO Binding Domain is a misnomer. Ten amino acids are unlikely to form an independently folding
domain and fluorescent-labeled 10-mers are poor binders of NEMO. The results of May et al. do point to
an important role for these amino acids in NEMO dependent IKK signaling, but no biophysical evidence
yet exists showing that the span of 10 amino acids itself can bind the NEMO protein.
**PEST** Region in primary sequence rich in *Pro, Glu, Ser,* and *Thr* amino acid residues

**Pfu** Thermostable DNA polymerase derived from *Pyrococcus furiosus*

**PMSF** Phenylmethyl sulfonyl fluoride

**RHR** Rel Homology Region

**SDS** Sodium Dodecyl Sulfate

**TNF** Tumor Necrosis Factor

**TNFR** Tumor Necrosis Factor Receptor

**Tris** Tris(hydroxymethyl)aminomethane

**UBHD** Ubiquitin Homology Domain

**Vent®** Thermostable DNA polymerase derived from *Thermococcus litoralis*

**On amino acids:** When amino acids appear within a sentence their italicized three letter representation is used. For example Serine is *Ser,* Threonine is *Thr,* Glutamate is *Glu,* etc.
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²A more verbose acknowledgment section may be viewed at the permanent location of this document: http://pobox.com/~dld/thesis/
³Doctor
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ABSTRACT OF THE DISSERTATION

Biophysical Characterization of NEMO and IKK2

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This dissertation describes the IκB Kinase 2 (IKK2) and NF-κB Essential Modulator (NEMO) proteins. Biophysical characteristics of N-terminal fragments of NEMO are described using methods that include circular dichroism (CD), equilibrium ultracentrifugation, and analytical gel filtration. Expression and purification of the N-terminal NEMO fragments are demonstrated along with the purification of exogenous IKK2 from an insect cell system. Purification of an active and stable fragment of IKK2 lacking 89 C-terminal amino acids is also presented.

Pulldown analysis and Isothermal Titration Calorimetry (ITC) experiments are implemented to demonstrate that NEMO 40-130 is capable of binding to the C-terminal 90 amino acids of IKK2, while smaller fragments including NEMO 40-90 and NEMO 60-120 are not. ITC data indicate that the association constant for NEMO 40-130 with GST-IKK2 665-756 is $4 \times 10^7 \pm 1 \times 10^7$ M. Further details are found within the text of this document.

Analysis of NEMO fragments by CD revealed characteristic double minima at 208 nm and 222 nm. These results form the first experimental confirmation of the software-predicted α-helical nature of the tested portions of NEMO. Thermal melts of NEMO 1-210 and 40-210 reveal a transition at roughly 40 °C. This confirms the qualitative observation that the N-terminal fragments of E. coli
expressed NEMO are unstable in a purified state. Thermal stability of NEMO 1-130 was too poor to be accurately measured by CD.

Equilibrium analytical ultracentrifugation experiments indicate that NEMO 1-130 may exist as a tetramer in equilibrium with monomers. Similarly, NEMO 1-210 appears form hexamers under the conditions tested. These data corroborate analytical gel filtration observations shared here that demonstrate a predominance of the tetrameric and hexameric oligomers for NEMO 1-130 and NEMO 1-210 respectively.

This work presents the first in vitro observations of N-terminal fragments on NEMO and their interaction with the IKK2 subunit. This work provides a foundation for further studies of IKK-complex structure and function.
1. Introduction

Thucydides observed immunity in his account of the Plague of Athens in the year 431 BCE [1], perhaps the oldest record of curiosity on the subject of immunity. Twenty-three hundred years later, contemporaries of Louis Pasteur learned that microbes cause disease and that immunity to them is conferred by our blood and specific cellular systems. Proteins called antibodies bind and assist our organism to neutralize and destroy microbes. Astrid Fagraeus showed in 1948 that developed B cells generate antibodies [2]. Decades later scientists applied the revolutionary tools of the 1980’s to outstanding questions about antibody production; B cells would contribute to the etymology of a new discovery. A protein family was named NF-κB for its association with specific DNA (the κ light chain gene enhancer) in activated B cells [11]. Although originally characterized as a constitutively nuclear factor specific to mature B cells, studies on NF-kappaB soon revealed that it is present in virtually all cell types but as an in-

![Figure 1.1: An antibody schematic, incorporating κ light chains.](image-url)
active cytoplasmic factor. Inactivation and cytoplasmic retention of NF-kappaB results from its association with a member of the IkappaB family of the inhibitor proteins. By regulating its interaction with IkappaB, NF-κB functions as an inducible transcription factor and a vital component of cellular stress responses. The chief component in determining whether NF-κB exists in its inactive cytoplasmic complex with IκB or as an active nuclear transcription factor is the multisubunit IκB kinase complex (IKK). Discovered in 1997, IKK functions as an integrator of upstream signaling events and downstream NF-κB activation.

A structural understanding of the NF-κB transcription factor system began to take shape in 1995 with the determination of atomic resolution x-ray crystal structures of NF-κB1p50 bound to DNA by Ghosh et al. [38] and Müller et al. [40]. These structures revealed for the first time how the dimeric NF-κB protein can recognize a specific DNA sequence contained within the promoter of a target gene. To do so, each NF-κB subunit contacts one half of the approximately 10 base pair target DNA with amino acid side chains contained within its amino-terminal Rel Homology Region (RHR). Subsequent structural studies on other NF-κB homo- and heterodimers have revealed the mechanism by which the conserved NF-κB RHR recognizes specific DNA target sequences. Additionally, x-ray crystal structures of the NF-κB1 p50/p65 heterodimer bound to IκBα have revealed how IκB proteins use an ankyrin repeat-containing domain (ARD) to target the NF-κB RHR and inactivate the transcription factor.

At the dawn of the twenty-first century, the field of NF-κB research has continued to expand. Completion of the human genome sequence confirms that the entire family of NF-κB subunit is encoded by but five genes: NF-κB1p50, NF-κB2p52, RelA_p65, c-Rel, and RelB. Aided primarily by genetics studies in mice and modern molecular tools like chromatin immunoprecipitation and RNA interference, NF-κB dimers composed of these subunits have been shown to participate in a complex network of gene expression signals. The family is now understood to be a crucial component for the body’s fast innate immune response, adaptive
immunity, and cell mortality.

Nature has conserved NF-κB pathway elements such as the Rel Homology Region (RHR) and ankyrin repeat containing inhibitors to assist organisms as diverse as flies, oysters, and people. Nature has also granted success to many pathogens that disrupt this important pathway. The IκB Kinase complex (IKK) is at a confluence of survival information that leads to NF-κB activation. Researchers unveiled its basic properties during the last three years of the twentieth century. This work provides new biophysical detail to the understanding of IKK2 and NEMO subunits of the IκB Kinase complex. This work moves the field incrementally closer to the atomic details of IKK function – details that will hasten the next phase of discovery in the realm of innate immunity.

1.1 NF-κB signaling

The canon of NF-κB signaling describes the rapid induction of DNA binding and gene transcription by this transcription factor. Current favor for the phrase classical pathway implies the inducible IκB Kinase activity purified with the IKK2 protein, dependent upon the NEMO protein. Cytokines like TNFα and IL-1, bacteria and bacterial products such as LPS, and viral flotsam like double stranded RNA in the cell exterior all activate cellular NF-κB by means of the classical pathway. Elevated DNA binding activity is notable at the earliest time-points after TNF stimulation of cultures, peaking at 30 minutes [91, 130]. Different NF-κB stimulants, and variations of stimulation time result in distinct patterns of proteins expressed by cultured cells, reflecting some of the complex network of events that occur to support an organism’s threat response.

The array of molecules that stimulate NF-κB and the function of those molecules whose abundance is regulated by NF-κB attest to its importance in cell, tissue, and organism survival. Activators and target genes were comprehensively reviewed in 1999 by Pahl [74], and a few are highlighted here. The
cytokines IL-1 and TNF are activators of NF-κB [19, 18]; they are also transcriptionally regulated by NF-κB [42, 22, 23]. Such an arrangement creates an amplifier for the innate immune response mediated by these cytokines.

*Mycobacterium tuberculosis* and *Listeria monocytogenes* are examples of bacteria that activate NF-κB [33, 31]. Many Gram-negative bacteria like the Rickettsia activate NF-κB [46] due to the Lipopolysaccharide (LPS) and lipoproteins that are unique to the cell wall structure of this class of bacteria. The host’s cellular response mediated by NF-κB includes the expression of cytokines that help recruit macrophages, neutrophils, and other intruder eaters from the innate immune system.

Figure 1.2 illustrates an example of classical NF-κB pathway signaling. At the exterior of the cell, an IL-1 receptor binds an IL-1 molecule [48]. TIR domains [83] of IL-1 receptors come together inside the cell upon extracellular IL-1 binding [104]. Adapter molecules like RIP and A20 in the case of TNFR [84] and MyD88 [107] in the case of most TLRs studied are recruited, and co-recruit the IKK complex and its upstream kinases. IKK activity is the result and it is notably dependent upon NEMO [84, 118]. Active IKK2 catalyzes the specific transfer of the γ-phosphate from ATP to Ser residues in the IκB destruction box (Figure 1.3 and 1, Figure 1.2) [11, 10]. The Ser phosphorylated destruction box is recognized by E3-RS_{IκB}, which transfers Lys73 branched poly Ubiquitin to Lys residues within the destruction box (2, Figure 1.2). The polyubiquitin IκB is a substrate for the 26S proteasome which proteolyzes the inhibitor (3, Figure 1.2), freeing NF-κB of IκB’s blockade of DNA binding and favoring NF-κB nuclear import (4, Figure 1.2).

The IκB Destruction Box (Figure 1.3) is the site of inducible phosphorylation [41] at the N-termini of IκBs. The motif may be tranferred to other proteins making them proteolytically sensitive with NF-κB stimuli [59]. A tool dubbed

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1The structures shown in Figure 1.2 are backbone cartoon renderings from the atomic coordinates. The scaling of one macromolecule relative to the others is approximate, and the IL-1 receptor structure has been mirrored rather than rotated in the IL-1α bound representation.
**Figure 1.2:** One way that NF-κB is activated is by extracellular cytokine molecules that bind to specific receptors and induce dimerization of the receptor proteins. Proximal intracellular receptor domains form a signaling surface that leads to the activation of the IKK complex. NF-κB:IkB cartoons are adapted from Figure 1.8. The Transcriptional Activation Domain (TAD) is drawn as a circle. The IkB Destruction Box is represented as a line at the N-terminus of IkB, and is labeled with a N. Dual phosphorylation of the Destruction Box by IKK (1) is represented by PP above the Destruction Box line. Branched ubiquitin modification (2) is represented by (UB)n. Poly-ubiquitin modified IkB is a degraded by the 23S proteasome (3), freeing an additional NF-κB NLS to interact with Importin proteins at the nuclear envelope (4). CBP et al. refers to the general transcription machinery which is co-recruited to κB enhancers upon NF-κB activation. See the text for a discussion of stimuli other than IL-1 which also initiate steps 1-4 outlined here.
the NF-κB superrepressor is an important contributor to the understanding of NF-κB signaling. The superrepressor is a mutant IκBα in which both IKK phosphorylatable resides in its destruction box have been mutated to Ala with its unreactive methyl group sidechain. This molecule is not broken down in response to IKK activating stimuli, and remains bound to NF-κB in the cytosol.

The crucial nature of IκB Destruction Box phosphorylation in NF-κB activation was shown with the superrepressor. The superrepressor has also demonstrated the IκB molecule’s important role in viral pathogenesis. Expression of the superrepressor in cultured Jurkat cells inhibits HIV1 replication [55]. It also blocks the ability of an alpha-virus to induce programmed cell death in cultured cells [56].

The NF-κB signaling paradigm presents a quick way for cells to react to potential hazards around them with a specific set of genes tailored by Nature to deal with those hazards. This simplified statement can be imagined to occur with different response speeds and longevities, different resultant proteins expressed, and variable amounts of cross-talk among other cellular signaling mechanisms. This description also provides an understandable explanation for how mammals have evolved to keep microbes at bay.

1.2 The NF-κB and IκB proteins

NF-κB is the enduring name for a family of transcription enhancement proteins. They were discovered as nuclear factors that associate with the κ light
**Figure 1.4:** The human NF-κB family members, including the viral protein v-Rel (a descendant of avian c-Rel), and the human IκB family members. At the bottom of the figure are the NF-κB:DNA (left) and IκB:NF-κB (right) structures described in Section 1.7 and Figure 1.8. Upper panel adapted from Bonizzi et. al. [108].
chain gene enhancer in activated B cells [10]. Twin κ light chains are present in about 50% of the antibodies a human will make during a lifetime. An enhancer region of DNA does not encode protein but increases the transcription of nearby genes. NF-κB was initially predicted to be essential to antibody production due to its presence at the κ gene enhancer region in activated B cells. Gene knockout studies prove that this is not strictly the case. Each NF-κB knockout however demonstrates an importance to aspects of immune function [66].

NF-κB was found in most cell types following its discovery in the activated B cell nuclei [29]. The inhibitor protein IκB restricts nuclear localization potential of NF-κB by masking the NLS of at least one protomer of NF-κB giving a cytosolic, inactive complex. The preformed inhibitor-transcription factor complex permits rapid response that does not require synthesis of new protein [10].

The human NF-κB family proteins are shown in the left panel of Figure 1.4, along with the avian virus v-Rel protein that is evolutionarily derived from avian c-Rel. Each contains a Rel Homology Region (RHR) that carries the functions of nuclear localization, dimerization, inhibitor binding, and DNA binding. The C-terminal domains of RelA, RelB, c-Rel, and v-Rel contain transcriptional activation function that turn on genes when the respective RHR binds to specific DNA sites in their enhancer regions. NF-κB1 and NF-κB2 are precursors of the proteolytically generated NF-κB1p50 and NF-κB2p52 proteins.

All members of the NF-κB family are capable of forming homo-dimers or hetero-dimers with other family members. NF-κB1p50 or NF-κB2p52 homodimers are transcriptionally inactive as are their full length forms NF-κB1p105 and NF-κB2p100. These proteins do not possess transactivation domains. The precursor proteins have inhibitor-like ankyrin repeat domains following their RHR. A Glycine Rich Region (GRR) links the RHRs to the ankyrin C-terminal domains and protects the former from the proteolytic processing that eliminates the C-

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2Langman and Cohn’s discussion of κ:λ ratios may interest the reader. [39]

3See [86, 92], and Figure 1.8.
terminal IkB-like domain.

The IkB proteins contain six or seven ankyrin repeats, and bind the RHRs of NF-κB homo- and hetero-dimers. This family is shown in Figure 1.4 and includes avian vRel alongside the human proteins. Recent work has uncovered the NF-κB, IkB, and IKK polypeptides in animals like dogs [120], cows [98], and trout [128]. The field will benefit as more are reported and become available to structural biologists.

1.3 The IkB kinase complex

The polypeptides that make up the IkB Kinase Complex include two kinase domain containing proteins, and a third protein predicted to be largely α-helical known as NEMO. The predicted domain organization of each of these proteins is shown in Figure 1.5. IKK1 and IKK2 subunits contain protein kinase domains and are 52% identical with one another overall. NEMO does not have greater than 24% identity with any protein of known structure, but does contain regions of high probability for forming dimeric coiled coils. Each of these proteins is described in more detail below.

1.3.1 The catalytic subunits: IKK1 and IKK2

Protein kinases catalyze the transfer of ATP's γ phosphate to the alcohol group found on the side chain of a Ser, Thr, or Tyr residue of a target protein. They make up 1.7% of the genes in the human genome [97], a reflection of their roles in diverse and dynamic processes that require specific control. The first protein kinase structure determined to atomic resolution was that of cyclic AMP dependent protein kinase (PKA) in 1991 [26]. PKA is a signaling molecule conserved among eukaryotic species that is sensitive to and crucial for the cell’s metabolic state. Its biochemical characteristics came to scientists’ attention during 1960’s [4] and a structural understanding of its regulation has only recently
Figure 1.5: Proteins, predicted domains and motifs of the IKK complex. Rectangle length and position are proportional to their corresponding amino acid numbers. Amino acid totals and molecular weight in kDa are shown on the right.
come to light [123, 131].

Researchers purified the inducible and specific IκB Kinase activity in 1997 and identified two kinase domain containing polypeptides [44, 50, 45]. The typically identified kinase domain homology spans about 270 amino acids. The N-terminal kinase domains of IKK1 and IKK2 and are 66% identical. IKK2 \(^4\) and IKK1 \(^5\) are 51% identical over their complete length. They are 66% identical when residues 1-300 are aligned\(^6\). Human IKK2 has 29% identity with the human protein kinase PKA\(^7\).

IKK1 and IKK2 phosphorylate two Ser residues within the Destruction Box of the IκB proteins shown in Figure 1.3. Experiments substituting Thr or Tyr residues at the IKK phosphorylation sites of human IκBα show a strong but not absolute preference for Ser over Thr [36].

The protein kinase catalytic core has a small –primarily β-sheet– N-terminal lobe and a larger α-helical C-terminal lobe. Magnesium, ATP, and the target amino acid of the substrate polypeptide bind at the junction of these lobes. The activation segment and the so called activation loop contained within this segment is a dynamic region of every protein kinase. It joins both lobes and along with the Gly rich loop acts as the gatekeeper of the active site. Inserts and evolutionary divergence separate many of the protien kinases in the activation loop region, however several amino acids are strictly conserved. The boundary amino acids for the activation segment are DFG and APE at the N- and C-termini of the segment respectively. This topic is nicely reviewed by Nolen et al. [87].

Work done by Manning and others documents the protein kinase subset of the human genome [97] and expands on the placement of different kinases into distinct groups and families [14, 96]. Groups are based upon kinases from different species that are apparent orthologs [5] sharing the same function. Families aggregate member kinases on the basis of function within a group. Fig-

\(^4\)Accession O14920
\(^5\)Accession O15111
\(^6\)BLOSUM62, Gap Penalties: Existence: 11, Extension: 1
\(^7\)Human PKA (Accesion NP997401) residues 1-290 aligned with full length IKK2 (O14920).
The Kinase Domain Activation Segment

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Group</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA</td>
<td>AGC</td>
<td>PKA</td>
</tr>
<tr>
<td>IKK1</td>
<td>Other</td>
<td>IKK</td>
</tr>
<tr>
<td>IKK2</td>
<td>Other</td>
<td>IKK</td>
</tr>
<tr>
<td>IKKε</td>
<td>Other</td>
<td>IKK</td>
</tr>
<tr>
<td>TBK1</td>
<td>Other</td>
<td>IKK</td>
</tr>
<tr>
<td>MEK1</td>
<td>STE</td>
<td>STE7</td>
</tr>
<tr>
<td>MEKK2</td>
<td>STE</td>
<td>STE11</td>
</tr>
<tr>
<td>MEKK3</td>
<td>STE</td>
<td>STE12</td>
</tr>
<tr>
<td>Sky1p</td>
<td>CMGC</td>
<td>SRPK</td>
</tr>
<tr>
<td>CDK2</td>
<td>CMGC</td>
<td>CDK</td>
</tr>
</tbody>
</table>

Figure 1.6: Family and group classifications are tabulated for the indicated protein kinases. The structural cartoon illustrating the activation segment of CDK2 1qmz [62] was adapted from a review by Nolen et al. [115]. The activation segment is highlighted in yellow (left) and shown in detail (right). [!] denotes identity among aligned sequences, [*] denotes similarity. Two IKK2 Ser residues are boxed in red that have been mutated to Glu to confer constitutive activity.
ure 1.6 illustrates the position of the activation loop relative to the small and large lobes of the CDK2 structure. It shows the grouping and families as determined by Manning et al., as well as a sequence alignment of chosen representative protein kinases.

An interesting region of homology with ubiquitin follows the IKK kinase domain toward the C-termini of the catalytic subunits. A stretch of 47 amino acids align with no gaps between IKK2 and the sequence of *ubq* (13/47 identities, or 27.6%; See Appendix E.2 on Page 106). The alignment of IKK1 with the same ubiquitin primary sequence shows fewer identities. The functional role of this domain is intriguing because of the important role that the ubiquitin protein and its branched polymers play in IκB degradation [36]. Little experimental investigation has brought light to this small fragment of the 100 kDa IKK proteins. The Ubiquitin Homology Domain *UBHD* is likely an important part of the IKK’s functional history, if not a structural element built from the ubiquitin archetype.

Experimental evidence indicates that the LZ region of the catalytic subunits are required for activation and homo and heterodimerization. The HLH region that follows C-terminally to the identified LZ on the other hand appears to be important to protein-protein interactions with upstream molecules [50]. Each of these motifs are predicted to assume α-helical secondary structure with high probability. Taken with experimental evidence, the leucine zipper of one IKK2 polypeptide will form a coiled-coil dimerization interface with another IKK2 protein, or form a heterodimer with the same structural element from and IKK1 protein. The helix-loop-helix portion may participate in a helical bundle configuration with similar tertiary elements from NEMO or other associated molecules.

In the year 2000, Sankar Ghosh’s laboratory identified an important stretch of six amino acids that are identically conserved between the two IKK catalytic subunits. Polypeptides that include this sequence, tethered to basic
amino acids that enable its movement across cellular membranes are able to block NF-κB signaling in vitro and in vivo [79]. Biochemical and in vivo evidence indicate that this polypeptide interrupts the interaction of IKK catalytic subunits with NEMO and the affiliated upstream signaling molecules that bind NEMO. The conserved amino acids from the catalytic subunits are shown in Figure 1.7. I use the term NBR: NEMO Binding Region for this peptide region. The NBR is important to the catalytic subunits ability to interact with NEMO. It can not comprise an independently folding domain, but is likely to be part of a larger fold, found at a surface where the subunits contact each other.

1.3.2 The NEMO subunit

NEMO is essential to NF-κB activation by the proinflammatory cytokines TNF and IL-1, bacterial products like LPS, as well as the diverse patterns recognized by the TLRs. Mutations and rearrangements of the ikkγ gene are often found in diseases of immune dysfuntion [110, 121]. The NEMO protein itself was identified in three distinct manners. Two groups sequenced it after biochemical purification of activatable IkB phosphorylation [57, 73]. Another encountered NEMO by its interaction with a viral protein [71]. The third method was a com-
plementation cloning experiment performed by Yamaoka et al. [60].

A portion of NEMO that spans about 75% of the protein is predicted with high probability to forr α-helical secondary structure. Figure 1.5 shows two dark lines spanning 100 amino acids each that are predicted to participate in dimeric, and possibly trimeric coiled-coil interactions. Multiple coiled-coil probability predicting programs inform that dimeric coiled coil is very likely while trimeric coiled-coiles are unlikely except in short stretches of amino acids. Unfortunately the current versions of coiled-coil structure prediction software are not yet able to anticipate the orientation (parallel versus antiparallell) nor the register or binding partner of a given stretch of coiled-coil α-helical structure.

The extensive roles of ubiquitin in NF-κB activation cycles are becoming better understood recently [124]. As early as 1996, it was appreciated that ubiquitin played both a role in activation of IKK as well as in degradation of IκBα. Studies in the laboratory of Zhijian ”James” Chen have clearly demonstrated how, as part of the classical NF-κB activation pathway, a unique protein-ubiquitin ligase attaches ubiquitin to the NEMO subunit of IKK. Wu et al. note that NEMO binds K63 branched polyubiquitin but not the more prevalent K48 branched polyubiquitins that are added to IκB.

1.4 Gene function in the NF-κB pathway

Table 1.1 lists the outcome of gene disruption in mice for some of the important proteins in the NF-κB signaling paradigm. RelA is the best studied and most abundant transcriptionally activating NF-κB family member. Lack of the RelA protein results in embryonic lethality at the 15th day of gestation\(^8\). The phenotype observed is wide-spread apoptosis of cells making up the liver. Researchers hypothesized that a lack of RelA rendered the fetal liver incapable of surviving a transient natural elevation of the cytokine TNF during development.

\(^8\)The mouse gestation period is about 21 days.
They tested this hypothesis by generating a double knockout that lacked the TNF receptor gene and the RelA protein. The result was a healthfully born fetus which succumbed to early death due to an ill-functioning immune system. The RelA\(^{-/-}\), Tnfr1\(^{-/-}\) phenotype supports a hypothesized role for NF-κB’s in protection from programmed cell death and pointed to the importance of NF-κB in both immune response and tissue development.

The Ikk2 knockout demonstrated a phenotype identical to that of RelA, and the creation of a Ikk2\(^{-/-}\), Tnfr1\(^{-/-}\) double knockout gave an analogous recovery wherein live-born mice live for a short time before succumbing to opportunistic infections. This result highlighted the requirement of the IKK2 subunit for NF-κB activation by TNF. These mice also provide primary cultured cells rich in experimental opportunities.

IKK1 was the first discovered kinase domain containing polypeptide of the IKK complex, yet cells cultured from Ikk\(^{-/-}\) mice are responsive to TNF, IL-1, and LPS. The phenotype for Ikk\(^{-/-}\) is embryonic lethality with pronounced in fetal development deficiencies. Analysis showed that hyperproliferation of specific cell types of the skin organ resulted in skin thickening and the hampering of skeletal development.

Distruption of the gene encoding the inhibitor protein IκB results in live-born mice which live for 7 days with extreme inflammatory problems, most notably associated with the skin [34]. Systemic inflammation results from the inability to attenuate NF-κB in the face of everyday exposure to pro-inflammatory factors in the environment.

The Ikk\(^{\gamma}\) (nemo) gene is located on the X chromosome\(^{9}\) [77]. Male fetuses receive only one copy of a disrupted Ikk\(^{\gamma}\) do not reach full term. They are stillborn with a similar phenotype to the RelA knockout and are non-viable by the 15th day of gestation due to massive apoptosis of the liver. Female double knockout fetuses suffer the same malady, but heterozygous females are born

\(^{9}\) Locus: Xq28
Table 1.1: Gene Knockout Observations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RelA&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Embryonic lethality (day 15) due to TNF induced apoptosis in liver. [35]</td>
</tr>
<tr>
<td>RelA&lt;sup&gt;−/−&lt;/sup&gt;, Tnfr1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Born alive, susceptible to infection. [65]</td>
</tr>
<tr>
<td>Ikk2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Embryonic lethality (day 15) due to TNF induced apoptosis in liver; Malfunction of NF-κB activation [72, 76]</td>
</tr>
<tr>
<td>Ikk1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Classical NF-κB pathway functional; Embryonic lethality due to epidermis development problems. [69, 70]</td>
</tr>
<tr>
<td>Ikk2&lt;sup&gt;−/−&lt;/sup&gt;, Tnfr1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Live born, susceptible to infection. [90]</td>
</tr>
<tr>
<td>IκBα&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>X chromosome locus [77, 81, 82]</td>
</tr>
<tr>
<td>nemo&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>Females live with <em>incontinentia pigmenti</em> like phenotype.</td>
</tr>
<tr>
<td>nemo&lt;sup&gt;−/−&lt;/sup&gt;,&lt;sup&gt;−/0&lt;/sup&gt;</td>
<td>Embryonic lethality (day 15) due to TNF induced apoptosis in liver.</td>
</tr>
</tbody>
</table>

suffering from a self-limiting disease which resembles human *incontinentia pigmenti* (IP). Incontinentia pigmenti patients develop inflamed skin lesions in lines occupied by an over abundance of keratinocytes which leave permanent lines once the affliction subsides. This mirrors the heterozygous mice (Females, *Ikkγ<sup>−/+</sup>* ) that exhibit these characteristic lesions, and other afflictions attesting to the analogy with human afflictions of IP patients.

### 1.5 IKK1, 2, and NEMO are required for canonical pathway signaling

The three proteins identified in a tightly associated 900 kDa IκB kinase activity are each required for pro-inflammatory induction of NF-κB. Certain responses to stimuli remain functional in cell cultures lacking IKK1 (addressed in review [105]) giving way to a pathway with a new name. It is referred to as the alternative NF-κB pathway and the main outcome is the proteolytic processing of NF-κB<sub>2,100</sub> to free heterodimers that contain RelB.
DNA binding by NF-κB upon IL-1 stimulation is intact with the elimination of the NEMO C-terminal Zinc finger. This same deletion eliminates activation by TNF however. Researchers were able to produce a dominant-negative inhibitor of NF-κB signaling by over-expressing a N-terminal portion of NEMO in cultured cells – rendering them unresponsive to either TNF or IL-1 [57]. Additionally a cell-permeable peptide [78] including the NBR was able to eliminate NF-κB signaling and co-IP of the catalytic IKK domains with NEMO [79].

1.6 NF-κB and disease

Transcriptions factors control the proteins expressed by a cell. Proteins and their networked functions constitute the identity of a cell. Nature has created NF-κB to quickly initiate the expression of specific genes that promote tissue and organism survival – sometimes by promoting proliferation, sometimes resilience to cell-death inducers, or production of survival factors. It promotes programmed cell death in other circumstance, but in all cases the expression of new proteins is transient, and except for the mature activated B cells, the NF-κB transcription factor resides transcriptionally inert in the cytosol ready for action. The inappropriate activation of NF-κB – namely, long term nuclear localization – can lead to disease, or may alternatively be a driven outcome of the diseased state which leads an organism to eventual collapse.

The correlation between NF-κB activation and cancer cells has been noted for some time. A fraction of solid tumors when analyzed are found to have constitutively nuclear NF-κB. The cRel gene locus is in a region prone to rearrangement and amplification. NF-κB2 and the gene Bcl3, an IκB family member are found in the same rearrangement-prone area. The avian virus encoded v-Rel is part of the aggressive tumorigenic outcome of infection of chickens. NF-κB is also activated by Herpes virus to prevent host cell apoptosis. IKK activity and IκB degradation are important for HSV suppression of apoptosis markers [111].
NEMO is an intriguing protein, not known to be a proto-oncogene per se, but it is experimentally implicated as a target of the Tax protein of Human T-cell Leukemia Virus (HTLV) which blocks NF-κB induced apoptosis of successfully infected cells. NEMO is also known to shuttle between the nucleus and cytosol [88]. SUMO modification, a process similar to modification by Ubiquitin, but occurring in the nucleus is known to happen on NEMO’s Lys–277 and Lys–309 in response to genotoxic stress [100].

The X chromosome locus of *nemo* leads to a variety of immune dysfunction and developmental disorders that are manifest in a X-linked manner. Ectodermal dysplasia describes a set of rare diseases that often occur along with other congenital disorders. Mutations and deletions that alter the Zinc Finger of the *nemo* gene result in increased susceptibility to many pathogens for the individuals carrying the gene and disease. Incontinentia Pigmenti is a skin developmental disorder that can result from mutations in the same region of NEMO. Incontinentia Pigmenti and Ectodermal Dysplasia patients suffer from opportunistic infections from a variety of pathogens due to the malfunctioning of NF-κB activation via IL-1, TNF, and Toll-like receptors that require viable NEMO [77, 81].

A large fraction of the mutations that have been found in NEMO linked diseases occur in CCHC Zinc finger at the protein’s C-terminus. It is known to be required for full IKK activation [95], and a deletion of the 4th through 10th exons is often observed removing the Zinc finger and then some from the C-terminus [110].

### 1.7 Structure in the NF-κB signaling paradigm

The first atomic resolution views on a NF-κB family member were determined in 1995 [38, 40]. Those models detail the NF-κB1p50 homodimer’s interaction with synthesized DNA oligomers based on the κB consensus sequence.
Table 1.2: IKK, NF-κB Signaling and Disease

<table>
<thead>
<tr>
<th>Ailment</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>Reviewed in [122, 125, 101, 94, 75]</td>
</tr>
<tr>
<td>HTLV</td>
<td>The Tax protein from Human T-cell leukemia virus interacts with NEMO [63, 68] to activate IKK [52, 58].</td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>IKK and NF-κB activity are elevated in breast tumors, NEMO 1-300 (dominant negative inhibitor of inflammatory NF-κB activation) reduces colony growth of a tumor cell line [89].</td>
</tr>
<tr>
<td>Leukemias and Lymphomas</td>
<td>The virus derived v-Rel protein causes lymphomas and leukemias in birds [67]. c-Rel gene rearrangement and elevated expression is found in 23% of diffuse large B-cell lymphomas. IκBα super-repressor inhibits the growth of activated B-cell like DBCL, implicating IKK [94].</td>
</tr>
<tr>
<td>Arthritis and Osteoporosis</td>
<td>A NBR containing peptide (NBD [79]) prevents the development of cells that resorb bone during chronic inflammation [114].</td>
</tr>
<tr>
<td>Ectodermal Dysplasia and IP</td>
<td>ED patients with severe immune dysfunction have mutations in nemo ZnF coding region. IP patients have compromised immune response, lower NEMO levels from usually one useful nemo gene.</td>
</tr>
</tbody>
</table>
**Figure 1.8:**  

A) Cartoon representations of the superset of structural observations of the IxBα·NF-κB (NF-κB1 DD, RelA RHR) complex from PDB entries *tikn* [53] and *nfi* [54]. The model was created by aligning the RelA dimerization domain from each structure. The result retains *tikn* coordinates for the N-terminal RelA domain, the NF-κB1 dimerization domain, as well as IxB beginning with the 3rd ankyrin repeat and ending with the C-terminal partial PEST domain observed in that structure [92]. The partial PEST which is unique to *tikn* is illustrated here in spacefilling mode. The N-terminal ankyrin repeats, RelA dimerization domain, as well as the RelA nuclear localization sequence unique to *nfi* are spacefilled. **B**Structure from A rotated 90° about the vertical axis as indicated.  

C) The NF-κB heterodimer (RelA*Δp65*:NF-κB1*p50*) bound to DNA, rendered from PDB entry *1vkx* [51].
NF-κB1p50 homodimers are transcriptionally inactive, but the structure gave great insight into the arrangement of its immunoglobulin folds, the C-terminal domain’s dimerization interacting surface, and the residues involved in specific base contacts at the κB site DNA. Moreover, the structure provided a basis for pursuing the structure of the transcriptionally active (and most abundant) NF-κB heterodimer RelAp65 - NF-κB1p50 bound to κB DNA [51]. The protein truncations performed in these studies retained strong mutually exclusive binding to IκB and κB DNA sites. The fact that the asymmetric κB site co-crystallized with the heterodimer demonstrated that NF-κB is able to structurally rearrange to appropriately match its sequence specifying chemical groups to DNAs with varying bend angles. This corroborated previous lower resolution bending angle and flexibility studies addressing κB sites of different target genes [37].

Two structures of IκBα bound to a NF-κB were solved within days of each other, and within months of the heterodimer-DNA structure [54, 53]. The NF-κB components crystallized by both research groups contained a NF-κB1p50 dimerization domain and a near complete RelA$_{p65}$ RHR. Figure 1.8 shows a hybrid of the output from both laboratories. The structure determined by Huxford et al. includes a portion of the acidic PEST region found at the C-terminus of IκBα. The PEST inclusive IκB makes more contacts with the the NF-κB heterodimer in this region at the DNA interface of the dimerization domains. This observation corroborated an experimental understanding of IκBα’s ability to remove NF-κB from DNA [80]. The N-terminal domain of RelA$_{p65}$ in the Huxford et al. structure also makes more extensive contact with its dimerization domain, and probably represents a more populated conformation in the cell. Jacobs et al. included a greater number of C-terminal amino acids from RelA$_{p65}$ and observed that polypeptide’s NLS in their electron density maps. The features that are unique to each structure are rendered as space filling atoms in Figure 1.8, while the common portions are backbone cartoons with one color per polypeptide. Rotations of 6° are stereo pairs in the figure.
1.8 Focus of study

The aim of this research is to add breadth to the understanding of IKK structure and function. Few structural insights have emerged since its discovery in 1997. Molecular biology and biochemical experiment have shown the important roles of IKK sequence elements. Gene knockout studies have clearly demonstrated the importance of NEMO, IKK1, and IKK2. Human diseases are caused by a lack of full length NEMO protein. Strong correlations exist between misregulation of IKKs and specific cancers. The structural elements of the IKK complex are unique to this important and specialized signaling complex. The arrangement of structural elements in three dimensional space will greatly inform an understanding of the complex’s regulation of catalysis.

Beyond determining the structure of the 600-900kDa IKK complex this work seeks the structure of relevant portions of the NEMO and IKK2 proteins that retain testable function. Protein expression and purification for biophysical characterization is a requisite intermediate goal. The resulting products must be testable for known biochemical function, and they must be reproducibly generated in a manner to be imitated by other researchers.
2. Purification and characterization of IKK2

2.1 Introduction

This chapter focuses on expression, purification and biophysical characterization of the IKK2 subunit. The main objective is to generate large amounts of highly pure full length IKK2 for structural work. While several reports demonstrated catalytic activity of IKK2 subunit purified from eukaryotic expression systems such as baculovirus and yeast, none of these reports showed purification of large quantities of IKK2 or its oligomeric property. All the work presented here was done with a constitutively active mutant of IKK2 where two serines in the activation loop were mutated to glutamic acids (Ser 177 Glu, Ser 181 Glu). These residues are important to the regulation of catalytic activity of IKK2 both in vitro and in vivo [45, 64]. Phosphorylatable residues at analogous positions in many other kinases play an important role in regulating catalysis.

Crystallization of IKK2 is an achievement that must precede its structure determination by x-ray crystallography. I purified IKK2 1-666 and grew crystals from the purified protein. The details of this process concluding my inability to reproduce the crystals are described in this chapter. The materials that I generated including full length IKK2 did allow me to make observations which are shared here.

Equilibrium experiments with an analytical ultracentrifugate permit
the measurement of the molecular weight of dissolved macromolecules. The instrument measures the radial position $r$ and a corresponding absorbance $A$ in cells that can be subjected to great, constant centrifugal forces. Figure 2.1 illustrates the internal arrangement of the instrument and includes the equations that allow the experimental determination of molecular weights (Equation 6.2).

Dynamic light scattering also gives information about the size of macromolecules and particles dissolved in a sample. The experiments do not permit a molecular weight to be determined without knowledge of the hydrodynamic shape of the solute, but they do give valuable information about the homogeneity of the solute. Crystallization experiments are more likely to succeed, and tend to be reproducible.

### 2.2 Results

The following sections describe results obtained while working with the IKK2 subunit alone. The production of full length and constitutively active IKK protein from insect cells is followed by the generation of the same protein lacking 89 C-terminal amino acids. Both are catalytically active. Preliminary
biophysical observations are shared. The production in *E. coli* of IKK2 fused to GST is also demonstrated. Catalytic activity of GST-IKK2 could not be detected.

### 2.2.1 Baculovirus expression of IKK2

I was able to express large amounts of His<sub>6</sub>-IKK<sub>2</sub>EE with an insect cell over expression system. The IKK2 baculovirus was obtained from our collaborator’s laboratory who had successfully purified IKK2 from baculovirus infected sf9 cells. I adapted their procedure of infection and purification which yielded approximately 10mg IKK2 after a single Ni<sup>2+</sup> affinity chromatographic step. Efforts to further purify the protein using different chromatographic steps reduced protein yield without much improvement in purity. Moreover, in many cases the protein appeared to be less pure after additional chromatographic steps as judged by SDS-PAGE analysis. Other smaller molecular weight proteins appeared suggesting degradation of IKK2 during purification.
2.2.2 Identification of a stable N-terminal domain of IKK2

Often a doublet in the crude soluble extract of sf9 cells expressing IKK2 was observed. The doublet remained after the Ni-affinity step. This suggested that the C-terminus of IKK2 is susceptible to proteolytic degradation. The top band corresponded to the expected size of the protein and bottom band appeared to be 5 to 10 kDa smaller in size. This was further confirmed by immunoblot analysis. Western blot using anti-IKK2 antibody was performed on freshly prepared IKK2 and an aliquot of IKK2 incubated for 3 days at room temperature. The intensity of the lower molecular weight band was enhanced when the sample was incubated at room temperature. The lower MW band seemed to correspond to the loss of roughly 80-90 C-terminal amino acids.

2.2.3 Baculovirus expression of IKK2 1-667

It was previously reported that the C-terminal region of IKK2 interacts with NEMO [79] and that several serines within this region undergo phosphorylation [64]. Further analysis of the sequence, location of the phosphorylation
sites, and secondary structure prediction led me to estimate that the sequence

\begin{align*}
660 & \text{IACSKVRGPPSVG} \ 671
\end{align*}

around residue 665 could be the site of proteolytic cleavage. A baculovirus expression construct of IKK2 encoding residues 1-667 was generated. Protein expression in virus infected sf9 cells was carried out as described in Chapter 6. The expression of this truncated IKK2 was qualitatively greater than the full length IKK2. Catalytic activity of truncated IKK2 was tested first. I found that this truncated protein was capable of phosphorylating GST-IκBα 1-54 \textit{in vitro}, as shown in Figure 2.4

\subsection*{2.2.4 Expression of IKK2 1-664 in \textit{E. coli}}

The region encoding residues 1 to 664 of IKK2 was also cloned into an \textit{E. coli} expression vector. This vector (pGEX 4T2) expresses heterologous proteins as GST fusion proteins. I undertook screens for expression conditions that would generate the GST fusion IKK2 in its soluble form. Figure 2.5 panels A and B show the expression of GST-IKK2 (1-664). Total expression of IKK2 was not very high and more importantly, only a small fraction of the total protein was soluble portion whereas most it remained in the insoluble fraction. Nevertheless, I partially purified the soluble fusion protein by GST affinity chromatography. Activity assay revealed that the \textit{E. coli} expressed soluble GST-IKK2 1-664 protein was catalytically inactive. It is important to note here that the \textit{E. coli} construct also had the \textit{Ser 177 Glu} and \textit{Ser 181 Glu} mutations. However, this apparently active forms of protein when purified from \textit{E. coli}, showed no catalytic activity. This suggests that the \textit{E. coli} expressed IKK2 is not properly folded and/or additional modification is essential for catalytic activity.
Figure 2.4: IKK2 1-667 produced in insect cells. A Ni\textsuperscript{2+} affinity, followed by S-75 size exclusion chromatography. B Kinase assay autoradiogram of \( ^{32}P \) labeled GST-IκBα 1-54.
Figure 2.5: A GST fused IKK2EE is expressed but inactive from *E. coli* cell lysates.  
A) Soluble and insoluble expression check side by side with GST protein fusion vector alone.  
B) Anti IKK2 western shows induction of IKK2 largely in the insoluble fraction.  
C) No activity is observed from the *E. coli* cell lysates, while Sky1p and IKK2EE isolated from recombinant baculovirus infected insect cells are active.
2.2.5 Sedimentation experiments using analytical ultracentrifugation

The polypeptide stoichiometry of pro-inflammation activated IKK complex remains an outstanding question in the field. I next wished to test the oligomeric state of baculovirus derived IKK2 through sedimentation experiments. Two types of sedimentation experiments are performed with an analytical ultracentrifuge. Sedimentation velocity experiments measure the rate at which macromolecules make toward the bottom of the cell compelled by centrifugal force. Larger molecules move more rapidly as the density of folded protein is greater than water and larger proteins represent a larger local concentration of the density differential. Two proteins of the same molecular weight can, however have different hydrodynamic properties due to their shape. This speed measuring method therefore cannot be used to learn the molecular weight of a macromolecule of unknown stoichiometry. Sedimentation equilibrium occurs when the centrifugal forces acting on the molecules move as many molecules toward the bottom of the cell as the force of diffusion moves the molecules back toward the top. The result is zero net movement of protein through the solution, and therefore the hydrodynamic shape of the proteins do not matter. In this state of equilibrium we can learn about the molecular weights of the proteins in solution. Equilibrium experiments have the disadvantage requiring several hours to reach the necessary equilibrium state, which, in turn requires the protein to remain stable on the equilibrium experiment time scale.

Analytical ultracentrifugation under equilibrium conditions was used to determine the particle size of IKK2. When an equilibrium exists among monomers and discrete oligomeric states, sedimentation equilibrium experiments may be analyzed to determine the respective association constants. Non-specific aggregates are observed as large particle behavior that departs from ideality when an attempt is made to model or fit the data to a molecular weight.

The results of the analytical ultracentrifuge equilibrium runs are shown
**Figure 2.6:** Full length His$_6$-IKK2$_{EE}$ exhibits non-ideal behavior for equilibrium experiments in an analytical ultracentrifugation at low speed (5,000 rpm). The buffer contained 1mM DTT, 20mM Tris pH 8.0 and 200 mM NaCl, and 500µg/mL His$_6$-IKK2$_{EE}$. Residuals from a fit to a single molecular weight yield an indeterminate number of protomers in the large average molecule observed in this experiment.
Figure 2.7: Full length His₆-IKK₂EE aggregates strongly at high pH, and is polydispersed with a predominant \( R_h \) of > 50nm (500Å) at low pH.

in Figure 2.6. There are problems with the data obtained with IKK₂EE. This protein appears to form a large aggregate and the data could not be properly fit into a reasonable monomer-multimer equilibrium. This observation alone could describe the non-ideal signature of the residuals shown in Figure 2.6.

2.2.6 Dynamic light scattering

I also tested the behavior of full length IKK₂EE with an instrument capable of measuring dynamic light scattering (see Section 6.4.3). The measurement of scattered light can be used as a tool to judge the presence of discrete
or randomly associated protein multimers dissolved in solution. This method uses the fact that multimers or large particles comprised of pure protein scatter light more strongly than small monomers do. Hence, the method is capable of distinguishing between the purity of polypeptides as observed by denaturing gel electrophoresis, and the purity of particle sizes that may result from association of the polypeptides in solution.

A major benefit of DLS experiments is that they are quick and one can recover most of the required sample. Successful experiments require centrifugation of samples to remove large undesired particles which scatter light more than the solute of interest. The results shown in Figure 2.7 were done after microdialysis into buffers prepared at pH 4.5 (acetate), pH 7.5 (imidazole) and pH 9.0 (BisTrisPropane). A trend of increasing aggregation at lower pH is apparent. This could be expected from a protein with a calculated isoelectric point of 6.5. The large aggregates appear to be near mono-dispersed. However, the particle size is extremely large. The estimated hydrodynamic radius of this large particle could accommodate as many as 200 dimers. The polydispersity observed at high pH could be the result of chemical crosslinking, or peptide backbone instability of the full length protomers. This could be a result of the purification process where they may be associated in large particles as observed in the imidazole condition. Regardless, these experiments taken together demonstrate that purified His\textsubscript{6}-IKK\textsubscript{2\textsubscript{EE}} alone, under the conditions tested, is a poorly behaved protein.

### 2.2.7 Crystallization of His\textsubscript{6}Flag-IKK\textsubscript{2\textsubscript{EE}} 1-666

Crystal trays were set up with His\textsubscript{6}Flag-IKK\textsubscript{2\textsubscript{EE}} 1-666. We obtained roughly 5mg of > 90% pure material with which we set up Hampton’s Crystal Screen, and Crystal Screen II. The first months following setup showed no crystals.

The crystals shown in Figure 2.8 was found after 15 months at 20°C. The Hampton Crystal Screen solution was mixed 1:1 with 12mg/ml protein. The
Hampton solution contained 70% MPD, 0.1 M Hepes pH 7.5. The protein sample contained 20mM Tris pH 8, and 200mM NaCl. There were three crystals in the drop and all three were carried to Argonne National Lab. Two of these crystals were tested for diffraction. No spots were observed even with long exposures. I failed to reproduce these crystals and therefore no conclusion of the material contained in the crystals could be made.

2.3 Discussion

Expression and purification of IKK catalytic subunits were optimized and reported previously. However, previous work used a single-step purification method. In this work, purification of IKK2 was further optimized using a multi-step purification method. The most significant outcome of these studies was identification of a core domain of IKK2. This protease sensitive fragment of IKK2 contained most of the structured domains, predicted by secondary structure prediction methods, located at the N-terminal portion. This fragment was expressed in baculovirus and purified using a procedure used for the full length protein. More importantly, this truncated fragment retained the catalytic activity. However, it lost the ability to interact with the adapter NEMO subunit.

A second critical finding is the complete lack of activity of IKK2 (1-666)
when expressed in *E. coli*. This fragment contained the phosphomimetic Glu mutations in the activation loop. Experiments carried out by others in the laboratory showed that full length or truncated IKK2 expressed in *E. coli* were all inactive. This suggested that either the protein was not properly folded or post-translational modification at other sites were also essential. To this end it is important to mention that most eukaryotic kinase interacts with heat shock proteins. IKK2 was shown to interact with the HSP 90 complex. It would interesting to test if active protein from *E. coli* can be derived when IKK2 is coexpressed with HSP 90.

Crystallization efforts of this IKK2 (1-666) were not successful. Our initial thought was that the presence of the tag at the N-terminus could be the reason why no crystals were obtained. Although, efforts were made to remove the tag by protease cleavage, they were not successful. Thrombin treatment removed the tag only from a small fraction of IKK2, and prolong treatment with the enzyme initiated cleavage at cryptic sites.
3. Purification and Characterization of Deletion Mutants of NEMO

3.1 Introduction

NEMO serves as an adapter connecting the upstream signaling module to downstream events. NEMO is an integral component of the classical IKK complex. However, its primary association is with the catalytic IKK2 subunit. The domain structure of NEMO has been extensively studied using cell based transfection and biochemical studies. Most of the in vitro biochemical studies address the central and C-terminal segments of the protein. NEMO is predicted to fall into an all-α classification by PROFsec [28] with over 76% of the sequence predicted with high probability to be α-helical. Secondary structure prediction indicated that a significant fraction of helical NEMO segments is capable of forming coiled-coiled structure [49]. The three distinct regions of high probability for coiled coil are illustrated in Figure 1.5. A region present in the N-terminus, a region present in the middle and a region in the C-terminus. The central region contains a recognizable LZ motif [57, 73, 60]. NEMO apparently shuttles between the cytosol and the nucleus, and it is modified at various sites. Functional significance of these modifications are becoming more clear. However, none of the modifications is observed within the IKK2 binding region. Alain
Israel and collaborators have performed extensive work on the central region encompassing residues 242 to 388 using chemical crosslinking, size exclusion chromatography and sedimentation by ultracentrifugation. They showed that this central region formed a stable trimer [106]. Cellular work with different deletions of NEMO reveal importance of these regions in cells. As mentioned in the introduction the N-terminal region of NEMO has been shown to interact with IKK2. Focus of this section is to biophysically characterize the IKK2 binding region of NEMO.

### 3.2 Results

#### 3.2.1 Generation of NEMO N-terminal deletion mutants (NEMO1-130 and NEMO 40-90)

Several published articles showed that the N-terminal domain of NEMO interact with IKK2. It was reported in 2000 that the C-terminal 100 amino acids of IKK2 could pull down NEMO 1-196 and 44-419, but interacted poorly with NEMO 86-419 [79]. Further studies showed that interaction with IKK2.
His\textsubscript{6} - NEMO 1-130  

His\textsubscript{6} - NEMO 40-90


Figure 3.2: Purified NEMO 1-130 and 40-90.
appeared to minimally require first 120 residues of NEMO [68]. I generated NEMO 1-130 deletion mutant in light of results that the C-terminally truncated NEMO can still bind the catalytic subunits [57]. This observation was further supported by the presence of a potential secondary structure boundary at residue 130. A shift in the coiled-coil propensity was also present near amino acid 130 as determined by multicoil [49]. A smaller NEMO fragment (NEMO 40-90) was prepared based on the notion that 44-419 retained most binding for IKK2 but 86-419 did not. Both fragments were expressed in E. coli as soluble proteins and the construction, expression and purification have been described in the Materials and Methods section. Purity of freshly prepared NEMO 1-130 and NEMO 40-90 is shown in Figure 3.2. A small fraction of both proteins exist cross-linked species and degree of cross-linking significantly enhanced after prolonged storage at -80 or -20 °C as shown in the bottom panel of Figure 3.2. Surprisingly, the cross-linking could not be reversed even after boiling with excess reducing agents.

We used these protein perform biophysical experiments. Three different methods were used to characterize these fragments; circular dicroism (CD), sedimentation using analytical ultracentrifuge and size exclusion chromatography.

Circular dichroism gives information about the asymmetric environment of the peptide bonds comprising the dissolved protein and can indicate the overall α, β, or random coil content intrinsic to the protein under the given experimental conditions. Analytical ultracentrifugation data reveals information about the molecular mass of the natively folded protein solute. Size exclusion chromatography provide molecular masses of these fragments from which oligomeric states could be suggested.

Circular dichroism spectrometers measure the ellipticity that results when right and left handed circular polarized light pass through a sample containing asymmetric centers. Peptide bonds strongly absorb UV light between
**Figure 3.3:** Circular polarized light [126]

**Figure 3.4:** Measurement of ellipticity, adapted from Adler et al. [6].
180nm and 230nm. Peptide bonds experience an asymmetric environment in a protein’s folded state due to the differences in polarity on the discrete sides of the otherwise mirrored symmetry of the NH–C–O bond. This results in slight differences in the extinction coefficient for right and left polarized light. Figure 3.4 depicts the ellipticity that circular dichroism spectrometers measure. In the left panel, left and right polarized light are absorbed equally. In the right panel, left polarized light is absorbed to a slightly greater extent than is right polarized light. The resulting vector is measured, and the process is repeated through a range of measurable wavelengths.

Early attempts to analyze NEMO 1-130 and NEMO 40-90 proteins to CD measurements resulted in poor data, partly due to the presence of Tris buffer, an organic molecule which absorbs strongly through the wavelengths of interest for the peptide bond. Furthermore, quality of proteins when used long after purification deteriorated. The purification scheme outlined in Section 6.2.2 was adapted to use phosphate buffer in place of Tris buffer. Figure B.3 shows the second step of purification of NEMO 1-130 using phosphate buffer. Fractions 3 and
Figure 3.6: One step Ni\textsuperscript{2+} column purification of the indicated NEMO 40-90 mutants.

4 from Figure B.3 were pooled and taken directly to the CD spectrometer to collect the data shown in Figures B.4 and 3.5. Similar purification scheme was also adapted for NEMO 40-90. Consistent with the prediction, far UV CD spectra of both these deletion mutants of NEMO show a double minima at wavelengths 208 and 222 nm characteristic of an \(\alpha\)-helical protein. Figure 3.5 demonstrates the double minima for NEMO 1-130. Additional CD data for NEMO 130, NEMO 40-90, and NEMO 40-90\textsubscript{c54a-c76a} are shown in Figure B.4 and Figure B.2 (lower and upper panel) respectively.

### 3.2.2 Generation of point mutants in NEMO 40-90

In addition to cross-linking, both NEMO 1-130 and NEMO 40-90 exhibited severe tendency to aggregation. It was thought that exposure of hydrophobic residues to the solvent might be the reason for these behaviors. To improve solubility of these proteins, several single and double mutatants were created in NEMO 40-90. Mutantions are listed in Figure 6.

Each mutant was purified in a one step Ni\textsuperscript{2+} affinity step. Figure 3.6
shows the outcome of three parallel Ni$^{2+}$ affinity purifications of the indicated double mutants Notably, neither of the Leu mutants exhibited the cross-linked band that appears in the wild type NEMO 40-90. Two Cys to Ala mutations were subsequently introduced as one appeared on the same face of a helical wheel as the LeuLeu and IleLeu did. These Cys to Ala mutants mimicked the behavior of the hydrophobic-diad double mutants. The study of these proteins was discontinued after we repeatedly noted no observable binding of IKK2 by the NEMO 40-90 wild type or mutant fragments, as described in detail in Chapter 4.

3.2.3 Generation of other NEMO deletion mutants

Severe problems encountered with NEMO 1-130 and NEMO 40-90 in purification and storage led me generate other deletion mutants of NEMO. It was hoped that other mutants might behave better in solution. A schematic map of full length and different truncated NEMO fragments used for this study are shown in Figure 3.7 Panel A. The new fragments were expressed in E. coli as poly-histidine fusion proteins as the previous ones and purified as soluble proteins. Distinct solution behavior of these fragments was noticed during and after purification. We define the qualitative solution behavior as a function of protein precipitation during concentration in a buffer containing 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5 and 3 mM DTT (10 mM 2-marceptoethanol). Figure 1B demonstrates the purity of these proteins. NEMO 1-210 and NEMO 40-210 are the two highly stable proteins that could be concentrated to over 20 mg/ml at different salt concentrations (50 mM to 500 mM NaCl) and pH (6.5-8.5). The smaller NEMO fragments were highly insoluble in solution displaying propensities to aggregate at 100 mM NaCl, 20 mM Tris-HCl, pH7.5. These smaller fragments also portioned into a high molecular weight component even under SDS (indicated by asterisks) as noted for NEMO 1-130 and NEMO 40-90 and shown in Figure 3.7. The ratio of monomeric form to the high MW protein was striking in case of NEMO 40-120 or NEMO 60-130 (compare lanes
Figure 3.7: A. Schematic map NEMO and the deletion constructs. ZF denotes zinc finger.  
B. SDS-PAGE profiles of purified NEMO fragments. The gel was stained by Coomassie brilliant Blue. [*] denotes possible NEMO related high MW components. C. Western blot analysis of the indicated NEMO deletion mutants.
5 and 11 to the rest). However, higher molecular weight bands of NEMO 40-120 and NEMO 60-130 appeared even in freshly prepared samples. The higher MW forms were also clearly visible for the NEMO 40-130 and NEMO 242-388 fragments. To test if these high MW species are related to NEMO, we carried out Western blot analysis of some of these samples shown in Figure 3.7 Panel C. NEMO 40-130 and NEMO 40-120 clearly showed that the upper bands were related to NEMO. The longer NEMO deletion constructs did not reveal any high MW band related to NEMO. The upper band of in the NEMO 1-130 sample was not visible as this sample was freshly purified. We excluded the possibility that these high MW species were dimers as boiling of the samples in the presence of high concentration of reducing agents could not convert them into monomers. The high MW species might be NEMO fragments covalently cross-linked to itself, or unknown proteins, or peptides.

3.2.4 Analysis of the secondary structure and foldedness of the NEMO N-terminal fragments

Circular Dichroism (CD) was used to determine the nature of the secondary structure of NEMO 1-210, NEMO 40-210 and NEMO 1-130 deletion mutants. Lower helical content of NEMO 1-130 is consistent with the shorter length of this protein.

I carried out thermal melting analysis of these fragment to test the folding stabilities of the N-terminal fragments by monitoring the change in ellipticity at 222 nm as a function of temperature at pH 7.5. Helical properties were retained in all three cases when the spectra were taken after completing a cycle of thermal denaturation by heating to 90 °C and renaturation by cooling to 10 °C. NEMO 1-210 and NEMO 40-210 showed a clear transition of helical content at approximately 40 °C. These experiments thus suggest that NEMO 1-210 and NEMO 40-210 are only marginally stable proteins with T_m of about 40 °C. The folding transition is a reversible process indicated by the identical spectra
Figure 3.8: CD Spectra and thermal melts of His$_6$-NEMO 1-210, (A. and B. respectively) and His$_6$-NEMO 40-210 (C. and D. respectively). [×] spectra taken at 10 °C, [•] spectra taken at 90 °C, [°] spectra taken at 10 °C following 90 °C data collection.
of these fragments before denaturation and after renaturation. We have also carried out similar structural analysis with NEMO 1-130. This fragment too displays helical structure. Unfortunately, melting analysis could not be done properly due to its sensitivity to aggregation.

3.2.5 Sedimentation studies of NEMO 1-210 and NEMO 1-130

To determine the oligomeric states of NEMO 1-210 and NEMO 1-130, these proteins were subjected to sedimentation experiments using analytical centrifugation under equilibrium condition at 4 °C. Sedimentation data were obtained at two different protein concentrations at a rotor speed of 17,000 rpm for NEMO 1-210 and 25,000rpm for NEMO 1-130. These data sets were fit globally by means of non-linear least squares program to different models; ideal single species, monomer-dimer, monomer-trimer, monomer-tetramer etc. The best model that data can be fit was monomer-tetramer for NEMO 1-130 and monomer-hexamer for NEMO 1-210. Fits shown in Figure 3.9 are based on these models. Stability of the fragments during the centrifugation run has been assessed by analyzing the samples after the run by SDS-PAGE, shown in Figure 3.10.

During a different sedimentation experiment with NEMO 1-130 I observed unusual behavior. The data could not be fit to any model. When the sample after the run was completed under SDS-PAGE I noticed degradation of the protein. Mass-spec analysis of the core stable fragment show that the remaining protein encompassed residues 54 to 130. The protein fragments identified after tryptic digest, SDS-PAGE separation and LCMS are shown in red in Figure 3.11.

3.3 Discussion

We initiated our work with NEMO in an effort to find fragments that functionally relevant complexes with IKK2. Although previous work showed
Figure 3.9: Sedimentation equilibrium data fit to a monomer-hexamer for His$_6$-NEMO 1-210 (A.) and a monomer-tetramer for His$_6$-NEMO 1-130 (B.). Data for NEMO 1-210 were recorded at 17,000rpm and data for NEMO 1-130 were recorded at 25,000rpm. The lines shown represent fits that modeled a monomer-hexamer equilibrium for NEMO 1-210, and a monomer-tetramer equilibrium for NEMO 1-130.
Figure 3.10: Assessment of the stability of three NEMO fragments during the course of a sedimentation equilibrium experiment.
NEMO 1-130

Matched peptides shown in **Bold Red**

<table>
<thead>
<tr>
<th>1</th>
<th>MNRHLWKSQ</th>
<th>CEMVQPSGGP</th>
<th>AADQDVGLGE</th>
<th>SPLGKPAMLH</th>
<th>LPSEQAPET</th>
</tr>
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<tbody>
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<td>ILRERCEELL</td>
<td>HFQASQREEK</td>
<td>EFLMCKFQEA</td>
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<tr>
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<td>KDLKROKQ</td>
<td>EALREVEHLKR</td>
<td>CQQQMAEDKA</td>
<td>SVKAQVTSSL</td>
</tr>
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<td>151</td>
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<td>EGRARAASEQ</td>
<td>ARQLESEREA</td>
<td>LQQHHSVQVD</td>
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<tr>
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<td>YDNHIIKSSVV</td>
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<tr>
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<td>EEALVAKQEV</td>
<td>IDKLKEEAEQ</td>
<td>HKIVMETVPV</td>
</tr>
<tr>
<td>301</td>
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<td>KLAEEKKELLQ</td>
<td>EQLEQLQREY</td>
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<td>QIHVMECIE</td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 3.11:** Peptide coverage of NEMO 1-130 proteolytic products. **Black:** NEMO 1-130 amino acids not found in any flying peptide. **Red (Grey):** Peptides derived from His6-NEMO 1-130 degradation band following analytical ultracentrifugation. **Light Grey:** NEMO amino acids 131-419.

the N-terminal region of NEMO was important for IKK2 binding, biochemical characterization of the NEMO N-terminal region was lacking. We generated two fragments based on studies done previously. We were able to generate large amounts of these fragments. Evidence for their poor behavior in solution include crosslinking in the presence of reducing agent, and variability in apparent molecular weight for proteins that appear to be pure by Coomassie stained SDS-PAGE. Once crosslinking has occurred in these protein samples it appears to be irreversible. The higher molecular weight bands remain after boiling in the presence of SDS detergent and amel reducing agent. The NEMO 40-90 and 1-130 appear to aggregate non-specifically. I was however able to collect CD spectroscopy date for these fragments. They exhibit double minima at 208nm and 222nm indicative of α-helical proteins. Software predicts an overall α-helical structure for NEMO. These CD results represents the first experimental confirmation of the software predictions. We made several other N-terminal truncation mutants of NEMO. A C-terminally extended version (1-210) is well-behaved in solution.
Secondary structure prediction methods indicated that the N-terminal region spanning residues 50 to 195 is alpha helical and a large portion of it may form coiled-coil structure. These observations strongly suggested that the arbitrarily chosen residue 130 is not the end of a protein domain that properly buries hydrophobic surfaces. Further deletion from or extension of the C-terminus resulted in dramatic differences in solution properties of these proteins. Further extension of the C-terminus to residue 210 produced a fragment that behaved as a folded protein without much exposed hydrophobic surface. As indicated from secondary structure prediction methods the first 40 residues did not play any significant role in influencing the protein solubility. When the minimal IKK2 binding fragment (40-130) was further deleted by 10 residues at the C-terminus or by 20 residues at the N-terminus, the smaller fragments showed much severe aggregation properties indicated by precipitation during concentration. Furthermore, these smaller fragments formed cross-linked oligomers that were stable under SDS-PAGE in the presence of 5 mM DTT. This suggests that these smaller fragments exposed residues that could undergo chemical reaction to unknown compound including another protein or peptide.

Careful optimization of purification led us to generate good quantities of NEMO 1-210 and NEMO (40-130). All four of the IKK2-binding fragments, NEMO 1-210, NEMO 40-210, NEMO 1-130 and NEMO (40-130), were used for further biophysical analyses. The present study has characterized the thermal stabilities of three NEMO N-terminal fragments by melting. Two longer NEMO fragments (NEMO 1-210 and NEMO 40-210 showed unfolding transitions around 40 °C. Considering globular proteins show higher thermal stabilities with an average unfolding transition at around 50 °C, these NEMO N-terminal fragments are thermally unstable.

We could not test the folding state of NEMO 1-130 because of temperature-sensitive aggregation. Based on our qualitative results that progressive deletion from both ends of NEMO 40-130 made the smaller proteins to form cross-linked
complex suggests that NEMO 40-130 at least adopts weakly folded conformation. Sedimentation and size exclusion chromatography experiments demonstrate that both NEMO 1-130 and NEMO 1-210 are oligomeric proteins. However, NEMO 1-130 appears to be a tetramer and NEMO 1-210 is a hexamer. It is unclear to us if these oligomeric states are physiologically relevant or artificial ensembles of protomers that relies on their concentration and solution conditions. Extensive studies have been done on the central part of NEMO 242-388, which has been shown to form a trimer. It is unclear to us if the reported trimer is a physiologic trimer. We suggest that the assembly of NEMO N-terminal fragments into higher oligomers is guided by differential and/or the weak but differential folding stabilities of these fragments.
4. IKK2 Binding by NEMO

4.1 Introduction

This chapter describes studies done on the binding interactions between NEMO and IKK2. It has been shown by in vitro GST pull down and cell-based experiments that a small segment of only 11-residues (ALDWSWLQTE) near the C-terminus of IKK2 is sufficient to bind NEMO. We showed previously that IKK2 has a proteolytically sensitive site near residue 665. It was thought that the entire C-terminus after the proteolytic cleavage site might form an independent domain or substructure. Based on this assumption this entire fragment would be better suited for binding studies with NEMO rather than a simple 11-mer peptide. We have expressed the C-terminal 91 residues long (665-756) segment of IKK2 as a GST fusion protein (GST-IKK2 665-756), which contains 11-residues long NEMO binding region (Figure xx). GST-pull down assays, size exclusion chromatography and titration isothermal calorimetry methods have been used for binding studies.

4.2 Results

4.2.1 The N-terminal region of NEMO interacts with the catalytic IKK2 subunit

We first broadly mapped the IKK2-binding region of NEMO by performing GST pull down experiments of GST-IKK2 665-756 with three large
**Figure 4.1:** Pulldowns with GST-IKK2 665-756, and controls of GST, and beads alone.  
B. NEMO fragments 40-120, 40-130, 40-210, and 60-130.
fragments of NEMO covering nearly the entire protein; NEMO 1-210, NEMO 242-288, and NEMO 302-419. As expected, GST pull down experiments demonstrated that only the N-terminal NEMO 1-210 fragment interacts with IKK2 Figure 4.1. The previous experiments showed that the N-terminal region encompassing the first 130 amino acids of NEMO is necessary for interaction with the catalytic IKK2 subunit. Furthermore, it was indicated that the binding region of NEMO might be located within a 50-residue segment from residues 40 to 90. We have generated four other NEMO truncation mutants, NEMO 1-130, NEMO 40-90, NEMO 40-130 and NEMO 40-210 and tested their binding for GST-IKK2 665-756. GST-pull down experiments reveal that except for NEMO 40-90, all other N-terminal fragments interact with IKK2 (Figure 4.1). These results identified NEMO 40-130 as the smallest NEMO segment capable of IKK2 binding. We further deleted 20 residues at the N-terminus (NEMO 60-130) or 10 amino acids at the C-terminus (NEMO 40-120). Although, NEMO 40-120 retained binding activity for IKK2, the binding activity appears to be greatly reduced compared to that of NEMO 1-210. NEMO 60-120 does not bind IKK2 with detectable affinity.

4.2.2 Oligomeric state of the IKK2 binding region of NEMO

I have further tested the oligomeric state of two N-terminal NEMO fragments, NEMO (1-210) and NEMO 1-130 by size exclusion chromatography on a Superdex 200 HR 10/30 column. Both proteins elute over a large volume, indicated by the broad peak seen in Figure 4.2. This may indicate that each of these fragments might be in multiple oligomeric states which are in equilibrium with each other. The centroid of elution peak allowed us to estimate the elution volumes of NEMO 1-210 and NEMO 1-130 at xx ml and yy ml, respectively. The molecular mass calculated from the standard curve suggest NEMO 1-130 is close to a tetramer whereas NEMO 1-210 is a hexamer in solution. These values are similar to those obtained from the sedimentation experiments.
**Figure 4.2:**

A. Chromatographic profiles NEMO, IKK2 and the complexes between NEMO and IKK2 fragments. BioRad standard contained a mixture of globular proteins with the indicated molecular weights.

B. SDS-PAGE profiles of the peak fraction of each samples injected (stained by Coomassie).

C. MW analysis of free proteins and complexes from the standard curve.
Table 4.1: Binding constants and thermodynamic properties of binding as determined by isothermal titration calorimetry. Each NEMO fragment shown in the table was loaded into the injector at a concentration near 50 µM. The cell within the calorimeter contained 5 µM GST-IKK2 665-756. Raw data, and individual binding isotherms are included in Appendix A.

<table>
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<th>NEMO</th>
<th>$K_a$ (M)</th>
<th>N</th>
<th>$\Delta H_{obs}$</th>
<th>$\Delta S_{obs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-90</td>
<td>Not Fit</td>
<td>Not Fit</td>
<td>Not Fit</td>
<td>Not Fit</td>
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<tr>
<td>1-130</td>
<td>1.197E7 ±7.8E5</td>
<td>1.5230 ±5.9E-3</td>
<td>-1.5600E4 ±91</td>
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<td>40-130</td>
<td>4.242E7 ±1.3E7</td>
<td>0.8325 ±1.2E-2</td>
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</tr>
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<td>40-210</td>
<td>7.613E7 ±6.4E6</td>
<td>0.6900 ±1.8E-3</td>
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<td>-1.097</td>
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<tr>
<td>1-210</td>
<td>1.112E7 ±1.7E6</td>
<td>0.7907 ±9.8E-3</td>
<td>-1.1730E4 ±253</td>
<td>-7.098</td>
</tr>
</tbody>
</table>

To test the stoichiometry of IKK2/NEMO complexes again size exclusion chromatography was used. To this end, we prepared a new IKK2 665-756 fragment as a poly histidine fusion protein. The size exclusion profile of His$_6$-IKK2 665-756 suggests that this fragment a dimer in solution. Complexes between His$_6$-IKK2 665-756 and NEMO 1-210 or NEMO 1-130 were formed by mixing the two proteins with excess of IKK2. The mixture was then subjected to size exclusion chromatography. The elution profiles of these two complexes are shown in Figure 4.2 Panel A. The calculated molecular masses of IKK2/NEMO 1-210 and IKK2/NEMO 1-130 complexes are 232 kDa and 103 kDa, respectively. 103 kDa is close to the expected MW of an octameric IKK2 665-756 / NEMO 1-130 complex (calculated MW of 108 kDa) where a tetramer of NEMO possibly binds to IKK2 665-756 dimers. The IKK2 665-756 / NEMO 1-210 complex most likely is a dodecameric complex where hexameric NEMO 1-210 binds three IKK2 665-756 dimers.

4.2.3 Binding affinities of the NEMO and IKK2 complexes

I have carried out ITC experiments to measure the observed binding affinity of fragments of NEMO to IKK2 (GST-IKK2 665-756) at 25°C in 100 mM
NaCl, 20 mM Tris-HCl, pH 7.5, 3 mM TCEP. ITC is ideally suited to study this binding interaction because it measures the binding directly by monitoring the heat change associated with the binding interaction and requires no modification or labeling of the proteins. ITC is also considered one of the most accurate methods of detecting equilibrium dissociation constants in the mM to high nM range. In addition ITC provides a direct measure of the observed binding enthalpy ($\Delta H_{\text{obs}}$) and the stoichiometry of the binding interaction ($n$). Binding isotherms were obtained by titrating five different fragments of NEMO (1-210, 40-210, 1-130, 40-130 and 40-90 with the GST fusion construct of IKK2 665-756 (Figure 5, Table 2). Binding was observed in for all constructs except NEMO 40-90. In all cases the binding stoichiometry was consistent with a 1:1 stoichiometry for all experiments. Given that the previous data showed that the oligomeric states of NEMO N-terminal fragments to be a tetramer or a hexamer and the IKK2 fragment to be a dimer, this means that the interaction must be that of a dimeric IKK2 with a dimeric NEMO. The data fit well to a model for a single set of binding sites suggesting that there were no coupled equilibria between the binding of the dimeric IKK2 and tetrameric or hexameric NEMO. The highest affinity interaction was for NEMO 40-210 and NEMO 40-130, suggesting that the regions 130-210 and 1-40 are not essential for binding and in fact led to a reduction in binding affinity in these experiments. In all cases the binding was accompanied by a favorable $\Delta H_{\text{obs}}$. For NEMO 1-210 the entropy change of binding $\Delta S_{\text{obs}}$ was unfavorable, leading to a lower binding affinity. For NEMO 40-210 the $\Delta S_{\text{obs}}$ was close to zero. For NEMO 40-130, $\Delta S_{\text{obs}}$ was favorable. The difference in the thermodynamic contributions to the overall free energy $\Delta G_{\text{obs}}$ for the complexes appears to be compensatory leading to a relatively small difference in the binding affinity of the different NEMO constructs for IKK2. The raw data and fit isotherms associated with the results shown in Table 4.1 are found in Appendix A.
4.2.4 Purification of the IKK2/NEMO1-130 Complex

A method to generate the complex between IKK2 and NEMO 1-130 was developed in earlier months of the work. As shown in figure xx, the complex remained stable for throughout the purification. However, it was noted that the method could not reproduced properly. As indicated earlier in this chapter, the major problem could have been NEMO 1-130, which is not a well-behaved protein. Current work in the laboratory is using NEMO 40-210 or NEMO1-210 or NEMO as the partner proteins for the IKK2/NEMO complex formation.

4.3 Discussion

Using qualitative binding experiments, I have shown the N-terminal region of NEMO binds IKK2. Using quantitative binding experiments, I have shown that a region spanning residues 40-130 contains IKK2 binding sites. First 40 residues of NEMO appear to play a negative role in IKK2 binding. The most striking results is the lack of increased binding affinity of NEMO fragments that rendered NEMO N-terminus to behave better in solution. The segment C-terminus to 130 does not contribute to IKK2 binding. Therefore, although this C-terminal region (130-210) interacts with the N-terminal segments (40-130) in manner to generate a folded domain as shown by CD melting analysis, the domain foldedness does not impact IKK2 binding. Interestingly, oligomeric states of IKK2 and NEMO fragments are complex in nature. It is unclear if the oligomeric states of these fragments revealed by gel filtration and AUC has any physiological significance. However, experiments present here clearly reveal that the basic unit of the NEMO/IKK2 complex is a dimer to dimer. In this regard, finding that IKK2 C-terminal region also forms a dimer is novel. Previous studies recognized only the leucine zipper domain as the oligomerization domain. Further experiments are needed to determine the true stoichiometry of the IKK2/NEMO complex. Finally, amy analysis do not reveal why IKK1
**Figure 4.3:** His$_6$-IKK2$_{EE}$ and His$_6$-NEMO 1-130 purification after adding pure *E.coli* derived His$_6$-NEMO 1-130 to baculovirus lysate containing IKK2 and subjecting them to anion exchange and Ni$^{2+}$ chelation chromatography.
Figure 4.4: The purification of IKK2 and NEMO 1-130 in complex by size exclusion.
fails to strongly interact with NEMO as suggested previously. One possibility is that oligomerization of IKK1 C-terminus might be different than that dimeric arrangement of the corresponding segments in IKK2.
5. Discussion

I described the structural features of IKK2, IKK2-binding region of NEMO and the interaction properties of the NEMO/IKK2 complex. My study furthered the understanding on the domain boundary of IKK2. Although no structural analysis has been carried out on this segment, the fact that it forms a dimer suggest that this the C-terminal segment spanning residues 665-756 form an independent module. IKK2 is active in vitro when the C-terminal domain (CTD) is removed.

While previous work identified N-terminal region of NEMO responsible for IKK interaction, the nature of this interaction remained elusive. We have defined the smallest segment of NEMO that retains full IKK2 binding activity, which encompasses residues 40 to 130. However, neither this fragment nor its N-terminally extended version (1-130) is well-behaved in solution characterized by their propensities to aggregate and chemical reactivity. Secondary structure prediction methods indicated that the N-terminal region spanning residues 50 to 195 is alpha helical and a large portion of it can form coiled-coil structure. These observations strongly suggested that the arbitrarily chosen residue 130 is not the end of a protein domain that properly buried hydrophobic surfaces. Further deletion from or extension of the C-terminus resulted in dramatic differences in solution properties of these proteins. Further extension of the C-terminus to residue 210 produced a fragment that behaved as a folded protein without much exposed hydrophobic surface. As indicated from secondary structure prediction methods the first 40 residues did not play any significant role in influencing
the protein solubility. When the minimal IKK2 binding fragment (40-130) was further deleted by 10 residues at the C-terminus or by 20 residues at the N-terminus, the smaller fragments showed severe aggregation properties indicated by precipitation during concentration. Furthermore, these smaller fragments formed cross-linked oligomers that were stable under the denaturing conditions of SDS-PAGE in the presence of 5 mM DTT. This suggests that these smaller fragments exposed residues that could undergo chemical reaction to unknown compound including with itself, another protein or peptide.

Careful optimization of purification ultimately led us to generate good quantities of NEMO 1-130 and NEMO 40-130. All four of the IKK2-binding fragments, NEMO 1-210, NEMO 40-210, NEMO 1-130 and NEMO 40-130, were used for further biophysical analyses. The present study has characterized the thermal stabilities of three NEMO N-terminal fragments by melting, monitored by circular dichroic signal at 222 nm. Two longer NEMO fragments (NEMO 1-210 and NEMO 40-210) showed unfolding transitions around 40°C. Considering globular proteins show higher thermal stabilities with an average unfolding transition at ∼55 °C, these NEMO N-terminal fragments are thermally unstable. We could not test the folding state of NEMO 1-130 because of temperature-sensitive aggregation. Based on our qualitative results that progressive deletion from both ends of NEMO 40-130 made the smaller proteins to form cross-linked complex suggests that NEMO 40-130 at least adopts weakly folded conformation. Sedimentation and size exclusion chromatography experiments demonstrate that both NEMO 1-130 and NEMO 1-210 are oligomeric proteins. However, NEMO 1-130 appears to be a tetramer and NEMO 1-210 is a hexamer. It is unclear to us if these oligomeric states are physiologically relevant or artificial ensembles of protomers that relies on their concentration and solution conditions. Extensive studies have been done on the central part of NEMO (NEMO (242-388), which has been shown to exist as a trimer. It is unclear to us if the reported trimer is a physiologic trimer. We suggest that the assembly of NEMO N-terminal frag-
ments into higher oligomers is guided by differential and/or the weak but differential folding stabilities of these fragments. Recombinant full length NEMO apparently forms a large molecular weight complex based on size exclusion chromatography experiments reported in literature. However, our current observations suggest that it is possible that full length NEMO in its free form might be an unstable protein. An unstable dynamic protein has a greater exposed surface area, which would allow it to interact with a larger number of proteins. Indeed, NEMO has been shown to interact with a large number of signaling proteins in addition to the catalytic IKK subunits such as RIP, A20, TRAFs, TRADD, TAX, and CBP, to name a few. We cannot exclude the possibility that wt NEMO exists as a free molecule is a single species with a distinct oligomeric state. It is interesting to envisage that the larger unfolded higher oligomer of NEMO folds into a conformationally acceptable “globular” form upon interaction with its cognate partners in the cellular milieu.

We have determined the binding affinities of the complexes between IKK2 665-756 and the NEMO N-terminal fragments. Although the mechanism is not clear we observed that the presence of the first 40 residues is inhibitory to IKK2 binding. The equilibrium association constants (K_a) of these complexes range from 1 – 10 x 10^7 M. This reasonably stable association explains why NEMO and IKK2 primarily coexist in resting cells. In the absence of any knowledge of cellular concentrations it is difficult to know the fraction of NEMO that may remain free in the cell. Unlike tight complexes such as IκBα/NF-κB p50/p65 heterodimer (low picomolar binding affinity), which ensures inactivation of all heterodimer, it is not unreasonable to assume that a fraction of NEMO can exist free. Indeed, NEMO is known to exist free of IKK2 in vivo that participates in the signaling of zenotoxxic stress. This apparently moderate stability of the NEMO/IKK2 complex makes it easier to envision a scenario where this complex allows the regulatory subunit to exchange with other proteins/factors to form different complexes or to undergo modifications that further strengthens
the complex formation thus regulating NF-κB activation pathway. In fact, the existence of a sumoylated or ubiquitinated forms of NEMO have been recently demonstrated in literature and have been show to significantly affect the NF-κB signal transduction pathway.

The stoichiometry of IKK2/NEMO complexes emerged from our study is intriguing. IKK2 665-756 is a dimer in solution. Our chromatographic study demonstrates that the tetrameric NEMO 1-130 binds to two dimers of IKK2. Hexameric NEMO 1-210 apparently binds to three dimers of IKK2. While these results appear to be inconsistent, a remarkable 1:1 molar ratio of binding is apparent. The fact that NEMO binding C-terminal domain of IKK2 is a dimer in solution, led to the suggestion that the primary mode of binding is a dimer of NEMO to a dimer of IKK1 (2:2). Moreover, the similar affinity of IKK1/ NEMO 1-130 and IKK2/NEMO1-210 suggest that the possible 2:2 binding is non-cooperative, each dimer:dimer binding is independent to each other. Interestingly, modification observed in the case of 40-130 does not affect its binding to IKK2. We suggest that regions within the N-terminal domain of NEMO provide IKK2 binding, oligomerization and modifications are mostly independent events.

Because both IKK2 and NEMO contain separate oligomerization domains which might influence the oligomeric state of the native IKK complex, we cannot conclude if the native complex is a hetero-tetramer (dimer-dimer). The calculated molecular weight of tetramer is ~300 kDa. This might be the basic unit of an IKK complex. This basic complex can undergo further self assemble forming an octamer (tetramer-tetramer) or a dodecamer (hexamer-hexamer) ranging the molecular weight from ~300 for a tetramer to ~600 for an octamer to ~900 kDa for a dodecamer. In induced cells, the molecular weight of IKK complex increases from ~600 to 1200 kDa. In induced cells, NEMO is modified by ubiquitin. It is possible Ub-modification-dependent stabilization of NEMO-IKK complex also stabilizes the higher oligomer.
This study is the first report of quantitative analyses of the binding interactions between a catalytic and the regulatory subunits of IKK. One of the key events that this study has not looked at is the lack of binding between IKK1 and NEMO. Considering the fact that the CTD of IKK2 and IKK1 are very similar in sequence, in particular the segment that binds NEMO, it is fascinating to know how NEMO discriminates against IKK1. Future work is focused to understand this.
6. Materials and Methods

Milligram quantities of pure proteins are essential for the success of most biophysical methods that are used to characterize proteins. Crystallization searches are maximally successful when a reliable protein source and a reproducible purification scheme is developed. The material must be demonstrably homogenous in its covalent and folded structure. This poses a problem for many important and dynamic regulatory factors of different mammalian cell types. NEMO is believed to be Ubiquitin modified [117, 119], SUMO modified [100, 112], and phosphorylated on multiple sites [99]. These post-translational modifications are likely to be essential to aspects of NEMO function, and dispensable in other cases. The main goal of the structural biologist is to isolate complete macromolecules in a state that can be functionally verified. Expression systems in *E. coli*, among many differences, lack the eukaryote’s protein folding, modification, and sorting machinery. Success stories of functional ectopic protein expression in bacteria abound, but those techniques don not suit all proteins for the reasons mentioned above. Biochemists and structural biologists for practical reasons focused on proteins present as significant fractions of the total protein from an organism’s tissues.

Structural studies of NF-κB and IκBα took advantage of bacterial systems for expression, even though sizable quantities of these proteins are exist in the cytosol. Mammalian tissues could presumably be used as the starting material for purifying these proteins. The IKK complex catalyzes the phosphorylation of IκB proteins and is expected to turnover many substrates in its active state.
It is accordingly expected to be present at a small molar fraction of its cellular IkB:NF-κB substrates. Researchers including our collaborators nevertheless used large volume HeLa cell cultures to perform a classical purification of an inducible activity and identified the associated polypeptides [45, 44, 50, 57].

In order to study the IKK complex, we introduced IKK2 and NEMO into *E. coli* and insect cell over-expression systems as illustrated in Figure 6. Each of these engineered over-expression systems has the potential to make our proteins of interest in high proportions relative to other cellular proteins.

The sf9 insect cell expression system is a robust method for production of exogenous proteins. By infecting the insect cells in culture with recombinant baculovirus, specific gene products can be overexpressed in a eukaryotic environment [8, 16, 113]. Our collaborators successfully produced active His<sub>6</sub>Flag-IKK2<sub>EE</sub> [61] using a toolkit that required homologous recombination of modified virus with co-transfected transfer IKK encoding DNA. This protein incorporated two *Ser* to *Glu* point mutations, each found in the kinase domain’s activation segment where the Glu residues mimic phospho-Ser (see Figure 1.6).

The aim of extensive crystallization experiments drove our lab to establish a cell culture facility to permit in-house protein production with baculovirus. (See Appendix D.) Concomitant with the installation of these resources we made *E. coli* expression vectors to further pursue production of the NEMO and IKK2 proteins. All materials needed for the bacterial work were available in the lab and a strong expertise in the methods was well established.

Success in recombinant protein expression necessitates the development of reliable and economical means of purification. As the principal aims of this study are the crystallization and biophysical characterization of NEMO protein constructs, the protein purification schemes were optimized to favor purity and homogeneity over sample yield.

Crystallization of proteins is a stochastic event requiring a material that is capable of spontaneously assembling into ordered three dimensional arrays.
Figure 6.1: IKK subunits discussed in this document; expressed in E. coli or baculovirus infected insect cells. From top to bottom: GST-IKK2EE a GST fusion for expression in E. coli; His6-Flag-IKK2EE 1-756 made with Pharmacia’s system and incorporating a Flag encoding N-terminal primer; His6-Flag-IKK2EE 1-666 the same system ending after the HLH motif; His6-IKK2EE 1-756 made with Invitrogen’s system, allowing for removal of the N-terminal tags by Thrombin; His6-NEMO1-130 the N-terminal 130 resides of NEMO.
While many techniques exist to empirically determine the suitability of a particular protein sample for crystallization, it is ultimately determined by the investigator through hands on experimentation.

The requirement of large amounts of highly pure and homogenous protein required for crystallography studies can provide a source of material that is well suited for characterization by biophysical approaches. Furthermore, a firm understanding of the biophysical and in vitro biochemical character of a protein can give insight in designing and screening potential targets for protein crystallization. Therefore, the two processes, protein crystallization and biophysical characterization, go hand in hand. The materials and methods used in expression, purification, crystallization screening, and biophysical characterization of the NEMO and IKK2 proteins are detailed in the pages that follow.

6.1 Molecular cloning and mutagenesis

This section addresses general DNA handling and manipulation techniques and resources. Specific primers and discussion of solutions are also presented along with the materials used in their execution.

6.1.1 DNA purification

The small and large scale methods for purifying plasmid DNA from bacteria were followed according to the first chapter of the Molecular Cloning manual compiled by Sambrook, Fritch, and Maniatis [20]. The phenol-chloroform purification step was always skipped. On some occasions Qiagen products [127], or BioRad products were used according their included literature. These consumer kits use the same alkaline lysis principals outlined Sambrook et al. with alterations in the steps used to remove protein and membrane contaminants from the DNA.

The Invitrogen Bac-to-Bac expression kit has the advantage of generat-
ing recombinant baculovirus in *E. coli* harboring a recombinase and a modified viral genome. This permits the recombination event to be performed in *E. coli* rather than in the more finicky insect cell system. The literature included with the Bac-to-Bac kit ([113], page 51) gives an alkali lysis protocol with steps that preferentially isolate the large (>100,000 base pair) recombinant viral DNA over the *E. coli* genomic DNA and the small (<6,000 base pair) helper plasmid DNA.

### 6.1.2 PCR

The polymerase chain reaction is an enabling technology that was first described in 1986 [9]. This work takes advantage of PCR methods [13], manuals which describe them [20], as well as mutagenesis kits [116] toward the end of subcloning IKK cDNAs, generating site specific mutants, and testing for the presence of specific sequences in putative recombinant baculovirus and *E. coli* expression plasmids.

Reactions were run in 50 µL volumes, in thin-walled tubes without an oil overlay. Some reactions were performed in 100 µL volumes with an oil overlay to prevent evaporation. Problematic reactions or reactions requiring long elongation and therefore long overall reaction times used the 100 µL volumes and an oil overlay.

Two DNA polymerase enzymes were used on different occasions through this work. The *Thermococcus litoralis* polymerase sold as Vent by New England Biolabs was used for most of the smaller subcloning tasks, while the *Pyrococcus furiosus* enzyme Pfu (and Pfu Turbo$^2$) distributed by Stratagene was used for large fragment subcloning such as the ~2,200 BP of IKK2.

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$^1$Earlier technology required double-transfection, and plaque purification to isolate potential recombinants.

$^2$Apparently a trade-secret change in the buffer condition used for that enzyme.
6.1.3 Point mutations

Point mutants, as well as adjacent double mutations were made according to the literature accompanying Stratagene’s Quickchange [116] mutagenesis kit. The system takes advantage of the high-fidelity polymerases such as Pfu to PCR amplify an existing recombinant expression vector while introducing the desired base pair changes with specifically engineered primers.

6.1.4 His\textsubscript{6}Flag-IKK2\textsubscript{EE} and His\textsubscript{6}Flag-IKK2\textsubscript{EE}\textsubscript{1-666}

The His\textsubscript{6}Flag-IKK2\textsubscript{EE} 1-666\textsuperscript{3} baculovirus was graciously made by Dr. Yi Chen then working in Michael Karin’s lab. We chose the C-terminal stop site on the basis of the following evidence. It is at the end of the HLH motif which may be a domain boundary. The Ser rich segment which follows it is thought to be involved in negative feedback by phosphorylation [64]. The stable C-terminal degradation product shown in Figure 2.3 is consistent with the loss of about 90 amino acids.

The His\textsubscript{6}Flag-IKK2\textsubscript{EE} recombinant baculovirus was made with the Pharmingen system [102] with primers that encoded a Flag tag. The Flag encoding bases were at the 3’ end of the bases coding for a thrombin cut site. The N-terminal primer is identical for His\textsubscript{6}Flag-IKK2\textsubscript{EE} and His\textsubscript{6}Flag-IKK2\textsubscript{EE}\textsubscript{1-666} as described by Zandi et al. [61]. The C-terminal primer for the full length construct is as described in the same article, and the primer used to create a stop codon at the position of Cys 666 is shown below. The recombinant plasmid used to introduce the IKK protein was pAcSG HT-a -b or -c, each bearing a different frame shift.

\[
\text{IKKb}_n\text{Flag} \\
\text{5’ AGCTATCGCGCCGACTACAAGGACGACGATGACAAAAGCTGGTCACCTTCC 3’ }
\]

\textsuperscript{3}We noted the residue number only after selecting a stop site. We chose not to let the number influence our selection. One feature of particular interest is the number that this papyrus assigns to the Beast: 616, rather than the usual 666. ... this is the earliest instance that has so far been found [129].
Table 6.1: Subcloning with complementary overhangs.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Consensus Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>5'G,G A T C C</td>
</tr>
<tr>
<td>BclI</td>
<td>5'T,G A T C A</td>
</tr>
<tr>
<td>EcoRI</td>
<td>5'G,A A T T C</td>
</tr>
<tr>
<td>MfeI</td>
<td>5'C,A A T T G</td>
</tr>
</tbody>
</table>

IKKb_c756

5'TGACTCCCCGGGTCATAGGCTGCTCC 3'

IKKb_c666

5'TGACTCCCCGGGTCATGAAGCAATCTTCAGG 3'

6.1.5 GST-IKK2_{EE1-664}

Both BamHI and EcoRI sites are present in the full length, and 1-664 IKK2 insert. Primers were designed which encoded BclI at the 5' end, and MfeI restriction at the 3' end of the resulting insert. The IKK2$_{S10A}$ template [64] was used for template IKK. As illustrated in Table 6.1, overhangs were generated upon digesting the insert with Bcl-1 and MfeI. These complemented pGEX-2T plasmid that had been double digested with BamHI and EcoRI. Protein expression checks are shown in Figure 2.5, along with a western blot. The kinase assay in the same figure demonstrates the lack of activity from the bacterially expressed IKK2 protein.

IKKb2n_bcl

5'GGACGCTGATCAAGCTGGTCACCTCCTG 3'

IKKb664c_mfe

5'GGACGCCAATTGCTCAGGAGATTC3'
6.1.6 GST-IKK\textsubscript{EE}665-756

IKKb665\textsubscript{n}\_bam 5'GGACGCGGATCCGTCCTGGTCTGTGCAG 3'

IKKb756\textsubscript{c}\_eco5 'GGACGCGAATTCTCATGAGGCTGCTCCAG 3'

6.1.7 His\textsubscript{6}NEMO 1-130

The fragment of NEMO including residues 1 through 130 was PCR amplified from pBK-CMV NEMO template DNA. A vector map is included in Appendix F. The primers used were:

IKKgn\_nde

5' GGAATTCCATATGAATAGGCACCTCTG 3'

IKKg130c\_bam

5' GCGCATGGGATCCTCATCTCTTCTGCTCCAC 3'

6.1.8 His\textsubscript{6}NEMO 40-90

IKKg40n\_nde

5' GGCAGCCCATATGCACCTGCCTTCAGAACAGG 3'

IKKg90c\_bam

5' GCGCATGGGATCCTCACTCCTTCTGCTCCAC 3'

6.1.9 His\textsubscript{6}NEMO 1-210 and 40-210

IKKgn\_nde

5' GGAATTCCATATGAATAGGCACCTCTG 3'

IKKg40n\_nde

5' GGAATCCATATGCACCTGCCTTCAGAACAG 3'

IKKg210c\_bam

5' CGGGATCCCTACTCCACGCTGCTGCCCCTG 3'
Table 6.2: Table of primers used to make single and double point mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>NEMO40-90−I71A+L72A</td>
<td>5′GGCAGAGCAACCAGGCTGCAGGAGGCGCTGCGAG 3′</td>
</tr>
<tr>
<td>NEMO40-90−I71A+L72A</td>
<td>5′CTCGCAGCGCTCCCGCGAGGCTGCTGAGCCTTCCAAGCCAGCC 3′</td>
</tr>
<tr>
<td>NEMO40-90−L79A+L80A</td>
<td>5′GGCTGGCTTTGGAATGCGCAGCTCCTGGCAGCC 3′</td>
</tr>
<tr>
<td>NEMO40-90−L79A+L80A</td>
<td>5′CCCTTGGCAGCTCCTGGCAGCCAGCCAGG 3′</td>
</tr>
<tr>
<td>NEMO40-90+H81A+F82A</td>
<td>5′GAGACCTTCCAGGCAGCTGAGGAGAATCAAGA 3′</td>
</tr>
<tr>
<td>NEMO40-90+H81A+F82A</td>
<td>5′CTTGTGATCTCTCCTCCAGTGCGCAGCTGAGGAGG 3′</td>
</tr>
<tr>
<td>NEMO40-90−C54A</td>
<td>5′GATTCTGCGGAGCGCGACAGAGCTTCTGCATTTC 3′</td>
</tr>
<tr>
<td>NEMO40-90−C54A</td>
<td>5′GAAATGCAGAAGCTCTCTGCGCGCTGGCAGAGAATC 3′</td>
</tr>
<tr>
<td>His$_6$NEMO40-90−C76A</td>
<td>5′GGCATGCGCAGCTCCTCTTGGCAGCCAGCCAGG 3′</td>
</tr>
</tbody>
</table>

6.1.10 His$_6$NEMO 40-90 Mutants

Mutants of His$_6$NEMO 40-90 were made according to Stratagene product literature [116], using the primers outlined in table 6.2.

6.2 Protein over-expression and purification

6.2.1 Verification of protein overexpression

Levels of protein expression were confirmed with small overnight cultures, as demonstrated in Figure 3.2.1. Multiple recombinant clones that had been confirmed by DNA sequencing were transformed into BL-21 DE3 E. coli. The cultures were grown overnight at 37°C, and induced with 0.5mM IPTG for two hours in the morning. Cells were pelleted in a microcentrifuge and placed on ice. Whole cell lysates were made by adding 20 volume equivalents of saline solution to the cell pellets, adding SDS PAGE sample buffer and boiling at 100°C for 5 minutes. Samples were cooled for 5 minutes, and then foamed and homogenized by rapidly drawing the liquid into a 2mL syringe through a 14 gauge needle.
6.2.2 Ni\textsuperscript{2+} chelation chromatography

All but two of the recombinant proteins described in this chapter incorporate a N-terminal polyhistidine\textsuperscript{4} tag to facilitate purification [17, 24]. Polyhistidine tags at neutral pH coordinate metal ions\textsuperscript{5} such as Ni\textsuperscript{2+}. An immobile phase having an affinity for Ni\textsuperscript{2+} can specifically purify the tagged protein. This affinity method is an invaluable early capture step for protein purification. The buffers used during column preparation, protein binding, and purification are listed below.

**Binding Buffer** 5mM Imidazole, 500mM NaCl, 20mM Tris HCl pH 8.0

**Wash Buffer** 50mM Imidazole, 500mM NaCl, 20mM Tris HCl pH 8.0

**Elute Buffer** 1M Imidazole, 500mM NaCl, 20mM Tris HCl pH8.0

**Strip Buffer** 100mM EDTA, 500mM NaCl, 20mM Tris HCl pH 8.0

**Charge Buffer** 50mM NiSO\textsubscript{4}

Two brands of Ni\textsuperscript{2+} affinity resin were used for an immobile phase. Invitrogen’s ProBond \textsuperscript{2+} resin and Qiagen’s Ni-NTA each use nitrilotriacetic acid chemical groups linked to agarose beads [12]. No experimental comparison was made, but qualitatively the two brands performed equally well.

To charge the immobile phase 5 volumes of charge buffer were passed over one wet volume of resin that had been rinsed in 3 volumes of water. The column was equilibrated with 3 volumes of binding buffer before the application of cleared cell lysate. Binding buffer was run over the bed and care was taken to rinse the upper portion of the column itself to remove crude lysate that would otherwise adhere to the walls and potentially contaminate the purification at a later stage. The bed was disrupted as little as possible during this rinse step. After two or three column volumes of binding buffer had passed through the

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\textsuperscript{4}Six sequential histidines at the N-terminus of each protein.

\textsuperscript{5}Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+} and likely their heavier, more expensive group metals.
resin three volumes of wash buffer were applied. Protein was eluted by adding a little as 1 and as much as 4 column volumes to the resin. In the case of columns having a bed volume of less than 5mL, the elute buffer was applied directly to the bed with a pipette.

6.2.3 His\textsubscript{6}Flag-IKK\textsubscript{2}\textsubscript{EE}, and His\textsubscript{6}Flag-IKK\textsubscript{2}\textsubscript{EE}1-666

A His\textsubscript{6}Flag-IKK\textsubscript{2}\textsubscript{EE} baculovirus made by our collaborators \cite{61,102} was amplified by adding low-passage\textsuperscript{6} baculovirus at a MOI of < 0.2 to monolayers of sf9 insect cells\textsuperscript{7}. The monolayers cultured in Grace’s Media supplemented by adding 10% by volume fetal calf serum, a 100x mixture of Penicillin Streptomycin and L-Glutamine, and subsequently filtering the media.

To express protein a MOI of > 1 was added to High Five\textsuperscript{8} suspension cultures. After settling cells at 5,000xg and removing the culture media, cell pellets were frozen at −80°C. Lysis was performed on ice in buffer composed of 20mM Tris pH 7.5, 200mM NaCl, 1% Triton X-100, 100 \textmu M PMSF, Sigma-Aldrich protease inhibitor cocktail diluted to 1x.

We decided to generate a recombinant baculovirus from the Invitrogen Bac-to-Bac \cite{113} for two main reasons. Non-IKK residues included the N-terminal His\textsubscript{6} tag (MetGlyHisHisHisHisHisHisGly), followed by a Thrombin site (LeuValProArgGlySer), amino acids encoded by the multiple cloning region, and followed by the primer encoded Flag tag (AspTyrLysAspAspAspAspLys) \cite{61}. Interluding residues included, this adds to 25 amino acids; 12 of which could be cleaved away with Thrombin. Secondly, we were experiencing difficulties with reproducible large scale insect cell expression and we believed that part of the trouble was due to favorable virus propagation of wild-type virus carried forward from plaque purification.

\textsuperscript{6}Earliest passage virus available after plaque purification. The plaque purification method greatly enriches the recombinant baculovirus, but does not guarantee 100% pure recombinants. As virus passage increases, revertant wildtype virus may be selected as it is not encumbered by the insertion of interest.

\textsuperscript{7}sf9\textsuperscript{[93]} cells are derived from the sf21 (IPLB-sf21-AE)\textsuperscript{[7]} insect cell line.

\textsuperscript{8}High Five is the name under which Invitrogen sells the BTI-TN-5B1-4 cell line\textsuperscript{[30]}.  

6.2.4 **GST-IKK$^{\text{EE}1-664}$ and GST-IKK$^{\text{EE}665-756}$**

The literature accompanying Amersham Bioscience’s Glutathione sepharose 4B was followed for these one step purifications.

6.2.5 **His$_6$-NEMO 1-130**

The PCR product was digested concomitantly with both the Nde1 and BamH1 restriction enzymes in 1x BamH1 enzyme buffer. The expression vector pET-15b was restriction digested in the same manner. The products were subjected to electrophoreses and visualized with Ethidium Bromide and UV light in a preparative$^9$ 1.5% Agarose gel. The fluorescent bands were excised with a sharp clean razor blade, and purified with a QIAquick gel extraction kit. The linear pET-15b and PCR fragment with complimentary ends were ligated with T4 DNA Ligase. The PCR fragment was in approximately 100 fold molar excess relative to pET DNA.

Our experiments showed that optimal expression of His$_6$-NEMO 1-130 resulted when protein expression was induced with < 0.2mM IPTG for 6-10 hours at room temperature. After reaching OD of 0.5 while growing at 37 °C cultures were allowed to cool before IPTG was added. Culture volumes were typically 700mL LB broth with 100 µg/mL Ampicillin in each of 3 baffled 4L Fernbach flasks. These were autoclave sterilized before inoculation with 100-500 µL of cells that had been grown in 1mL cultures for 12 hours or less.

Highly competent JM109 *E. coli* were transformed with 1 µL ligation product for 50 µL thawed cells and plated overnight on LB Agar plates with 100 µg/mL Ampicillin. Ten colonies were picked to inoculate 5mL LB Amp$^{10}$ cultures. DNA was purified as described in Subsection 6.1.1, and submitted for sequencing. BL-21 DE3 *E. coli* were transformed with the same DNA and whole cell expression checks were performed. Figure 3.2.1 shows the expression

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$^9$Four wells were fused together with a small amount of clay before inserting the comb.

$^{10}$Sterilized LB media supplemented with 100 µg/mL sterile Ampicillin.
experiment; The expected molecular weight of the protein, including the N-terminal His tag is 17.4 kDa. DNA sequencing confirmed that all clones checked correctly coded for His$_6$-NEMO 1-130. Clone 3 was used for all subsequent experiments.

### 6.2.6 SR-IkB$_\alpha$

The SR-IkB$_\alpha$ protein incorporates the 13 C-terminal residues from Npl-3$^{11}$ at the N-terminus of IkB$_\alpha$. The protein was purified as described by Phelps et al. [80]. It is a viable substrate of both Sky1p [87] and IKK2.

### 6.2.7 cDNA sources

IKK2 PCR products were generated from a pRcβ actin plasmid which contained the EST amplified by Zandi et al. [50] as shown in Figure F.1. A variant [64] which harbored 10 Ser to Ala mutations in the region following the HLH motif was used for GST-IKK2$_{EE}$1-664 discussed in 6.2.4.

### 6.2.8 His$_6$-IKK2$_{EE}$, His$_6$-NEMO 1-130 complex

NEMO 1-130 was purified in one step by Ni$^{2+}$ chelation as described in subsection section 6.2.5. A total of 10mg of this protein was added to 100mL crude His$_6$-IKK2$_{EE}$ cell lysate at 1-2mg/mL, and was immediately diluted to 200mL on ice, with cold A buffer(20mM Tris-HCl pH 8 50mM NaCl). PMSF was supplemented by adding 100 µL of a 0.5M stock. The mixture was filtered through a 0.4 µm filter and subsequently loaded by gravity onto a 20mL bed volume FastQ column. The resulting flow rate was approximately 1mL/min.

The column was washed with 2 column volumes of low salt buffer containing 20mM Tris-HCl pH8 and 50mM NaCl. A gravity driven gradient from 50mM NaCl to 1M NaCl was applied spanning 5 column volumes with 20mM Tris-HCl 1M NaCl as the second buffer.

$^{11}$A substrate of Sky1p, the splicing kinase in yeast [87].
Fractions containing an IKK2 band as judged by coomasie stained SDS-PAGE, and later confirmed by western blot, were pooled and diluted 1:3 with Ni\(^{2+}\) Binding Buffer. This solution was applied to a 2mL Ni\(^{2+}\) column, then washed and eluted as described above. The eluate was buffer exchanged with size exclusion buffer in a Centricon YM-30 concentrator, and the final volume was brought to 1-2 mL for injection onto a Superdex200 column packed in a Pharmacia 16/60 casing. Size exclusion buffer contained 20mM Tris-HCl pH 8, 200mM NaCl and 1-2mM DTT.

### 6.3 Crystallization

Initial crystallization trials included sparse matrix screening [25] with commercially available kits[103]. Consistency of protein expression or capture was a major impediment, but sufficient quantities of His\(_6\)Flag-IKK2\(_{EE}\), His\(_6\)-IKK2\(_{EE}\), and His\(_6\)Flag-IKK2\(_{EE}\) 1-666 were obtained on a few occasions. When quantities permitted 96 iterations of 10 µg or more IKK2 per drop, we set up the Hampton Crystal Screen, or Crystal Screen II. Well volumes were 500 µL. Hanging drops were used consistently on silinized square glass cover slips. Drops were mixed (1 µL protein):(1 µL) well solution by adding well solution to the pre-placed protein drop and pipetting 1 µL up and down three times. Cover slips were sealed on 24 well plates with Corning vacuum grease, in some cases mixed 1:3 warm water : grease. Plates were labeled with the date and experiment information, and maintained at 20 °C in a Fisher Scientific air-flow maintained incubator. Crystallization drops were monitored by inspecting each drop at 200x magnification with a Olympus stereomicroscope fitted with two polarizing filters that could be rotated relative to eachother to verify birifringance in the drops.
6.4 Biophysical methods

6.4.1 Isothermal titration calorimetry

ITC experiments were carried out at 25°C on a Microcal VP instrument. NEMO and IKK2 proteins were prepared immediately prior to the experiment, by size exclusion chromatography on an S-75 column with eluant consisting of 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 3 mM TCEP. In a typical ITC experiment, twenty 15 µL injections of 50 µM NEMO were made into a 5 µM solution of GST-IKK2_{665-756} in the cell. Experiments were performed in duplicate or triplicate with different protein preparations. Heats of dilution of NEMO into buffer were recorded in a separate experiment and subtracted from the binding data. Isotherms were analyzed using the Origin software (Microcal) as described elsewhere and fit to a model for a single set of binding sites [21]. The NEMO protein concentrations used to generate the figures included in Appendix A. were as follows. 5.72 µM GST-IKK2 665-756 was titrated with 51.5 µM NEMO 1-210. 4.92 µM GST-IKK2 665-756 was titrated with 47.9 µM NEMO 40-210. 3.64 µM GST-IKK2 665-756 was titrated with 51 µM NEMO 40-130. 4.0 µM GST-IKK2 665-756 was titrated with 40 µM NEMO 1-130. 4.0 µM GST-IKK2 665-756 was titrated with 40 µM NEMO 40-90. The results of these titrations are summarized in Table 4.1.

6.4.2 Analytical ultracentrifugation

The UCSD Department of Chemistry and Biochemistry maintains a Beckman Optima XL-I analytical ultracentrifuge in its Biophysics Instrumentation Facility. The machine is equipped with interference optics and UV/Vis spectrophotometric functions connected to an automated data collection system. The data collection software *Data Acquisition Version p4.5b © 1998* coordinates multi-day experiments and directs changes in temperature and speed along with data collection.
The XL-I was used to analyze IKK2 alone and with separately purified NEMO 1-130 added at varied molar ratios. Each cell required 100 µL of protein with an optical density between 0.05 and 0.25 for the wavelengths chosen. The pure complexes exemplified in Figure 4.4 yielded insufficient quantities for these 100 µL cells. The experiment shown in Figure 2.6 was performed in 20mM Tris HCl pH 8.0, 200mM NaCl. The absorbance data were collected at 280 nm, and the rotor velocity was 5,000RPM.

Data were analyzed according to equation 6.1 by setting $v = 0.73$ mL/g for all proteins studied, and $\rho = 1.0$ mL/g for all buffers. The buffer used was typically 20mM Tris HCl, 200mM NaCl. A single molecular species exhibiting ideal behavior results in the apparent molecular weight $M_{app}$ is related to the molecular weight by equation 6.2 which includes a second virial term (and coefficient, B) that approximates non-ideal behavior at high protein concentration c.

$$M_{app} = \frac{2RT}{(1-\nu\rho)} \omega^2 \times \frac{d(ln c)}{dr^2}$$  \hspace{1cm} (6.1)

$$M_{app} \approx \frac{M}{(1 + BMc)}$$  \hspace{1cm} (6.2)

### 6.4.3 Dynamic light scattering

A Protein Solutions DynaPro systems was used to gather light scattering data for His$_6$IKK2$_{EE}$ alone after microdialysis into different buffers. The microdialysis method is outlined in section 6.6.1. Dialysis tubing with pore sizes yielding a 30 kDa cutoff [product name] was soaked in water before a two sequential soaks of 5-10 minutes in the target buffer. The three buffers uniformly contained 200mM NaCl, one containing 20mM BisTrisPropane at pH 9, another 20mM Imidazole at pH 7.5, and the last contained 20mM Acetate at pH 4.5. Three 200mL buffer exchanges were made at 1 hour time intervals at room temperature with stirring at about 100rpm. Each dialysis button contained 250-
300 µL at approximately 1mg/mL. Samples were centrifuged at 30,000×g, room temperature, for 5 minutes before being added to the apparatus for light scattering measurements.

6.4.4 Circular dichroism spectrometry

The Circular Dichroism Spectrometer used in this work was an Aviv Model 202 outfitted with a NES Lab CFT-33 refrigerated circulator. Data collection software \textit{cds.exe} reported a file version of 1.0.0.1. The version found in the online help document was 2.73, 6/8/2000.

The Aviv model 202 records data in units of millidegrees. The following equation was used to convert to peptide molar ellipticity units $\theta$.

$$\theta = \text{millidegrees} \times \frac{1 \text{ degree mg}}{1 \text{ millidegree g}} \times \frac{10 \text{ path lengths}}{\text{cm}} \times \frac{\text{cm}^3}{\text{mg protein}} \times \frac{17377 \text{ g}}{\text{mol polypeptide}} \times \frac{150 \text{ peptides}}{\text{polypeptide}} \times \frac{1 \text{ mol}}{10 \text{ decimol}}$$ (6.3)

Equation 6.3 reflects His$_6$-NEMO 1-130 that has a calculated molecular mass of 17,377 Da for 150 amino acids. The concentration of 0.1 mg/ml was inverted and inserted into the $\text{cm}^3 ÷ \text{mg protein}$ term. These were used to convert the data shown in Figure B.4 to molar ellipticity units ( $\theta$ ) (see Figure 3.5) required by the k2d de-convolution software [27].

The k2d software is described by Andrade \textit{et al.} [27]. It uses secondary structure content from known structures and corresponding CD spectra in the range of 200–240 nm to train a neural network algorithm. The software reports α helical, β sheet, and random coil content such that the sum equals 100%.

Spectra were taken sequentially at 10 °C, 90 °C, and again at 10 °C for the fragments shown in Figure 3.8. Thermal melts were subsequently recorded for fresh samples of each fragment. The thermal melts were monitored at
222 nm. The temperature ramping rate used was 5°C/minute, and data was collected using a 2 second averaging time. For these figures the y-axis units used were $\varepsilon$ which is related to $[\theta]$ by Equation 6.5. Units of $\varepsilon$ are easier to conceptualize. They report the difference in the molar extinction coefficient for left and right polarized light for the given wavelength (Equation 6.4).

$$\varepsilon = \varepsilon_L - \varepsilon_R$$  \hspace{1cm} (6.4)

$$\varepsilon = 3298 \times [\theta]$$  \hspace{1cm} (6.5)

6.4.5 Fluorescence measurements

A Jobin Yvon-Spex FluoroMax-2 model 1971\(^{12}\) was used to collect intrinsic fluorescence enhancement, as well as fluorescence polarization data. The machine was outfitted with a NESLab RTE-111 refrigerated circulator, and the automated L-Format Polarization accessory equipment. The servo drive systems of the L-Format Polarization accessory were inoperable. A method was devised which locked the incident polarizing filter into the servo’s frozen position, and then marking the parallel ($\parallel$) and perpendicular ($\perp$) position for the emission polarizer for manual manipulation \([109]\). An estimate of $\pm 1^\circ$ö is reasonable for the positioning error. Systematic errors may have been introduced. The emission filter was moved as infrequently as possible by alternatively measuring $\parallel$ followed by $\perp$, and subsequently $\perp$ followed by $\parallel$ for the sample that followed.

Samples were illuminated with the source light centered around 480 nm, and having a 4 nm slit width. Fluorescence emission was monitored at 90 ° by scanning from 460–560 nm in 5 nm increments. The region of 460–500 was included to assess the light scattering of the sample, and assure that scattering would not interfere with the observation of Fluorescien emission near 520 nm. Data was recorded manually and entered into an excel spreadsheet for analysis.

\(^{12}\)The fluorometer was built in 1996.
Free label was generated by reacting the succinimidyl ester 5FAM-SE for two hours with 100 mM Ammonium Acetate, and diluting the probe to a similar optical density as the labeled peptide with the same buffer as used for the peptide dilution. Measurements of $\perp$ and $||$ fluorescence intensities were recorded in triplicate to determine $G$, a correction factor (See the polarization principles appendix of the Jobin Yvon manual [85]) shown in equation 6.6, where $I_\perp$ and $I_{||}$ are the perpendicular and parallel intensities respectively, measured for the free probe.

$$G = \frac{I_\perp}{I_{||}}$$ (6.6)

The polarization $P$ described in equation 6.7[85, 3] was implemented in a spreadsheet after subtracting the blank values. Blank samples were prepared and measured in triplicate.

$$P = \left( \frac{I_\perp - GI_{||}}{I_\perp + GI_{||}} \right)$$ (6.7)

### 6.5 LCMS

A facility for LCMS experiments is overseen by Dr. Elizabeth Komives in association with the UCSD Superfund Basic Research Program Protein Characterization Core. The facility, directed by Justin Torpey, permits in-gel digestion and application of nano-mole quantities of protein for analysis on the LCMS machines.

Proteolysis was performed on excised SDS-PAGE bands. 100 µg excised bands were washed with 50% ACN and carbonate buffered water alternately. After acetonitrile washes by adding carbonate buffered Trypsin to 2 ng/µL and incubating overnight at 37 degrees C. The reaction was stopped by lowering with TFA to 0.01%.

Samples were applied to the integrated QSTAR XL system which in-
corporates a Vydac C18 analytical column (75 µm x 10 cm). The flow rate for reverse phase separation was 200 nL/min. The system’s MSMS feature was implemented, and data analyzed using its Information Dependent Acquisition software. This software searches protein sequence databases to identify peptides which may correspond with the mass peaks observed during the experiment. The software’s settings (Switching Criteria) were as follows:

- With charge state: 2 to 4
- Which exceeds: 30 counts
- Switch after: 1 spectra
- Exclude former target ions: 300 seconds
- Ions Tolerance: 100.000 ppm
- Ignore peaks within: 6.0 amu window

### 6.5.1 Analytical size exclusion chromatography

Size exclusion chromatographic analyses were performed on a calibrated analytical gel filtration column (Superdex200 HR 10/30; GE Heath Sciences). Samples applied to the column using approximately 100 µg of each protein shown in Figure 4.2. The column was equilibrated with a buffer made of 20 mM Tris pH 8.0, 100 mM NaCl, 3 mM TCEP. The complexes between His-IKK2 and NEMO1-210 or NEMO1-130 were formed by mixing the two proteins with excess IKK2. The protein mixtures were kept in ice for one hour before loading onto the column.

The column was calibrated using BioRad standards. The mixture of globular proteins include molecular weights of 670 kDa, 158 kDa, 44 kDa, and 17 kDa. Elution volumes \((V_e)\) were determined by peak integration with Pharmacia’s Unicorn software. Column void volume \((V_v)\) and total volume \((V_t)\) were determined by injecting Blue Dextran and vitamin B12 respectively. A linear regression of the \(K_{av,13}\) versus Log(MW) is shown in Figure 4.2.

\[ K_{av} = \frac{(V_e-V_v)}{(V_t-V_v)} \]
6.6 Miscellaneous methods

6.6.1 Microfuge tube cap dialysis

To dialyze 200–300 µL of protein into a new buffer background, the following procedure was followed. An eppendorf tube was cut with a sharp blade just below the cap’s protrusion into the tube with the cap closed. The bottomless tube was opened, and the top was cut loose. Protein sample was placed in the cap and carefully covered with a 4 cm² piece of dialysis tubing that had been rinsed in water and soaked in the target buffer. The bottomless tube was placed over the dialysis tubing and cap and gently pressed to close over the cap and seal the protein sample in the cap. This assembly was placed in 100 to 500mL beakers containing the target buffer. Air bubbles were removed by agitation if they were captured between the external tube wall and the external face of the dialysis tubing. Internal bubbles were minimized. Buffers were stirred at 50–150rpm and for 1–10 hours between changes for dialysate.

6.6.2 Amino acid sequence alignment

Protein sequence alignments were performed for a variety of reasons during this investigations. The software used to generate figures in this document include the Basic Local Alignment Search Tool provided by the NCBI [132], and the CLUSTALW [32] program which is packaged with the EMBOSS software suite. The sequence alignments shown in Figure 1.6, and other multiple protein sequence alignments not shown in this document were made using the CLUSTALX program [47], version 1.83 compiled for Mac OS 10.4. FASTA file formats were used for input. The Gonnet 250 Protein Weight Matrix was used with a pairwise gap penalty of 10.00 [0–100] and a gap extension penalty of 0.10 [0–100]. Input sequence data were entered into the Psi-BLAST [43] web utility found at the NCBI website [132], also in FASTA format.
A. Isothermal Titration Calorimetry Data

Figure A.1: Raw ITC data for NEMO 40-90.
Figure A.2: ITC binding experiments: Raw data and isotherms for NEMO 1-210 and 40-210.
Figure A.3: ITC binding experiments: Raw data and isotherms for NEMO 1-130 and 40-130.
B. Preliminary CD spectra for NEMO 1-130 and NEMO 40-90

The data shown in Figure B.1 were collected by Rashmi Talwar and formed part of a manuscript submitted for review in May of 2004. The first specific criticism from Reviewer 1 follows:

An attempt to establish the structural integrity of IKK-g N-terminal fragments is made by measurements of its CD and 1D NMR spectrum. While the NMR experiments are well-executed, the primary problem with Far-UV CD spectra is that their measurements were not made under the optimal conditions. The authors used a cuvette with a large path length (1 cm) and low protein concentration (3-10 µ/ml) in a buffer (20 mM Tris, 100 mM NaCl) which is known to give a strong solvent absorbance particularly in the deep UV (below 250nm). Therefore all unusual CD profiles, as the authors seem to recognize, can only be due to absorbance problems of the buffer.

I used this guidance and my own speculation to tune the CD experiments as follows. First, I believed that the extensive and irreversible crosslinking that I observed with SDS-PAGE could contribute to the strange spectra that Rashmi had shown. Oxidized Cys sidechains are known to absorb UV below 260 nm. The samples could be expected to demonstrate circular dichroic signal if a disulfide bond were to absorb strongly in a chiral environment of the folded protein. This led me to create the Cys 54 Ala Cys 76 Ala mutant. Its preliminary CD spectrum is shown in the upper panel of Figure B.2. The shape of the observed spectra...
curve was extremely sensitive to concentration. The data shown in Figure B.2 are the machine’s ellipticity values, \( \theta \) (millidegrees). The data were collected with a 1 mm pathlength Hellma QS cuvette at 20 °C. Protein concentration was approximately 1 mg/ml and 0.33 mg/ml for the double mutant shown in the upper panel, and 1mg/ml for wild type NEMO 40-90 shown in the lower panel.

The conclusion that I made was that the strange spectrum was not due to an absence of crosslinking. The NEMO 40-90 \textit{Cys 54 Ala Cys 76 Ala} double mutant contained no Cys residue, exhibited no crosslinking, and gave a spectrum very similar to the wild type protein under the conditions that I tested. I did not reproduce the spectrum shown in Figure B.1 as I had refined the methods and found the fingerprint of an \( \alpha \)-helical protein.

During this same period I was unable to reproduce the paper’s binding studies for NEMO 40-90. My conclusion therefore was that NEMO 40-90 is an \( \alpha \)-helical protein, and I have no evidence that this structure is functionally relevant. On the other hand, I was able to demonstrate that NEMO 1-130 was able to bind the catalytic subunit by pulldown experiments and refined fluorescence experiments adapted from the manuscript.

With confidence that NEMO 1-130 binds the IKK2 catalytic subunit, following the reviewer’s guidance above, and noting that human NEMO 1-130 contains 3 Cys residues, I decided to perform the rapid purification of NEMO 1-130 shown in figure in Figure B.3. I used a phosphate buffer in the place of Tris, and loaded higher concentration protein (1 mg/ml) into 1 mm path length cell. As observed in the subsequent figure (Figure B.4), this binding-verifiable protein also contains significant amounts of \( \alpha \)-helix.
Figure B.1: Early NEMO 40-90 CD spectrum.
Figure B.2: CD spectra for NEMO 40-90\textsubscript{wt} and and NEMO 40-90\textsubscript{c54a-c76a}. 
**Figure B.3:** Coomassie stained SDS-PAGE demonstrating the purification of His$_6$-IKK$\gamma$ 1-130 through Ni$^{2+}$ and AEX (Q) steps. Phosphate buffer was used to minimize UV absorption during CD experiments that were encountered in previous experiments with this protein.

**Figure B.4:** CD spectra and thermal melts of His$_6$-IKK$\gamma$ 1-130. The left panels were performed with the pooled fractions 3 and 4 shown in Figure B.3. The right panels were performed with pooled fractions 5 and 6.
C. Fluorescence results

Figure C.1: Fluorescence enhancement of a fluorescein labeled NBR peptide monitored as NEMO fragments or IxB δ concentration was varied.
Figure C.2: Fluorescence polarization of fluorescein labeled NBR peptide monitored as NEMO fragments or IxB δ was varied.
D. Establishment of a Cell Culture Lab for Exogenous Protein Expression with Baculovirus

At the outset of this project our collaborators in Michael Karin’s lab had generated active IKKα and IKKβ in reasonable quantities for some biochemical analysis. The author’s efforts under the instruction of Dr. Yi Chen, then at the Karin Lab, saw their method scaled to produce quantities as high as 5mg of one-step purified protein. The samples were measurably active and approximately 90% pure as estimated by Coomassie stained SDS-PAGE. The procedure monopolized the baculovirus expression resources of Dr. Karin’s lab. At the same time, a structural biology lab neighboring our own was interested in initiating mamalian and insect cell expression systems. The author took on the responsibility of selecting equipment and outfitting a cell culture facility that would be shared by Partho Ghosh’s lab and Gouri Ghosh’s lab. The facility was moved and expanded under the expertise of Dr. Alexander Hoffmann in February of 2004. It is presently located on the 3rd floor of UCSD’s Natural Sciences Building.

The startup cost estimate is shown in Table D.1. High-value items included an over-under water jacketed incubator needed for mammalian cell culture, a larger and cheaper air jacketed incubator for insect cells, one laminar
Table D.1: Estimated initial expenses for establishing a cell culture facility.

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</table>
flow safety cabinet, and an inverted objective lens microscope. The equipment was installed in a vacant room on the 6th floor of Urey hall in the Fall of 1999.

Preferred conditions for a culture room call for positive room pressure such that net airflow passes into the room through a HEPA filtered duct. One attribute shared by all laboratory spaces on the 5th and 6th floors of Urey hall at that time was extreme negative pressure apparently caused by the large flow of air into the chemical safety hoods. A chemical safety hood was present in the cell culture room and was not removed or blocked off when the biological safety cabinet was installed. The room pressure was negative as could be noted by the main door's strong tendency to pull shut. Recurring contamination was certainly due to a variety of reasons; this is likely one of them. Negative room pressure brings air, dust, and other airborne particles with it into the cell culture room from the less clean spaces around it. To properly finish the room its overhead air–duct should have been outfitted with a HEPA filter and the incoming airflow through it should have been adjusted such that a slight positive pressure was established in the room.

Expertise, training, and confidence are essential when bringing new methods and technologies to a laboratory. The initial users of the facility, author included, had some success with the cell culture facility and baculoviral expression in particular. Troubleshooting complex, difficult to isolate, and time consuming problems encountered in eukaryotic cell culture are beyond the aspiring expert’s capabilities however. The set of initial users included a post-doctoral student with experience in a supported cell culture facility as its most confident user.

In 2004–2005 the cell culture facility increased in size and moved to a designed–for–the–purpose room in the Natural Sciences Building. The new member of the shared facility was a new principal investigator (Dr. Alex Hoffmann) who had extensive background in mammalian cell culture. Any problems that remain in the consistency of IKK complex protein expression appear to be
isolated to the difficulties inherent to expressing these proteins.

Two kits that facilitate the molecular biology behind recombinant baculovirus protein expression were used in this work. The protein expressed in the Karin lab was done with a Pharmingen [102] kit which relies on homologous recombination in double–transfected insect cells as its fundamental trick. At the time that the author was beginning the molecular biology for new protein construct expression Invitrogen had developed a new method which obtained 100% recombinant baculovirus DNA from recombination in *E. coli*, followed by singly transfected insect cell cultures. In addition to ease, a major advantage of the latter is the lack of non-recombinant baculovirus recombination. Non-recombinant viruses are favored with increased viral passage requiring a process known as plaque purification to obtain high-titer virus, and thereby regularly expressible protein.
E. Sequence Alignments

E.1 IKK Catalytic subunits aligned with PKA

IKK2*------------------------MSWSPSLTTTQTCGAWEMKERLGTGGFGNVIRWHNQETGE
IKK1*------------------------MERPPGLRPGAGGWPWEMRERLGTGGFGNVCLYQHRELDSL
PKA**MASNSSDVKEFLAKTEDFLKWESEPQAQNTAHLQFERIKTLTGTGSGRMLVKKETGN 60
*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..* :..*:..*:..*:..*
IKK2*QIAIKQCR---QELSPRNRRWCEIQIMMRTHVPVVARDVPEGMQNLAPNDDLPLLAME
IKK1*KIAIKSCR---LELSTKRREWCEIQIMMKLNHANVVKACDVEELN-ILHNDVLPLLAME
PKA**HYAMKLDQKKVQKQIEHTLENYKLQAVNFPLFKLEFS-------FKDNSNLYMVME 114
*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..* 

IKK2*YCQGGDLRKYLNQFENCCGLREAGILTLLSDIASSALRYLHENRIHRDLKPNIVLQQGE
IKK1*YCSGGDLRKLKNPKENCCGLKESQILSLSDIGSGIRYLMKHIHRDLKPNIVLQQGE
PKA**YVPPGEMF5HLRRIG---RFSEPHARFYAAQIVLTFEYLHSLEYRDLPENLIDIQG 171
*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..* 

IKK2*QRLIHKIIDLYAKELDQGLSLTSCSTVGLQYLAPELLEQQKTYTVTDYWFSFTLAFECIT
IKK1*GKIIHIIKIDLYAKDVQDGQSLTCSSTVGLQYLAPELFPENKPTATNDYWFSFTLAFECIT
PKA**----YIQTVTDFGFAKVRKVQ---RTWTLCGPEYLAPEILKGNKAVWALGVLIIYMMAA 236
*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*

IKK2*GFRPFLNPWQVWHSVKRQKSEVDIVVSEDNLGTVKFSSSLPYPPNNLNSLAEILLEKWL
IKK1*GYRPFHLQQTFTWEHIKIKKDPKCIIFACEEMSGEVRFSSHLQPNLSCRLVPEMNWL
PKA**GYMMFAD-QPIQIYEKIVS----------------------GKVQIFPSHSSD 267
*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*:..*

IKK2*QLMLNWPQRQG--TDPTYGPNGCFFKALDILNLKLVHILNMVTGTIIHTYPVDTEDESQES
IKK1*QLMLNWDPOQRRGVPDPTLQKQPCFVLMHILNKIVHLNMMTSAKISFIPLLPPDESLHS
PKA**-------------------------267
IKK2*LKARIQDGIPEDEQDQLLEAQLALIPDKPATQCSIDGKLNÉGHTLDMDLTVLFDNSKI
IKK1*LQSRIERETGINTGSQELLSETGISLDPKRPASQCVALD-----VRGCSYMYVLFDKSKT
PKA**-------------------------267

104
E.2 Ubiquitin aligned with IKK2 and IKK1

IKK2

\text{1UBQ}^* - MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLLIFAG\text{--}\text{---}
\text{IKK2}^* - KALDDILNLKLVHILNMYGTIHTYPVTEDESLQSLKARIQQDTGPEEDQELLQEAGLA

\text{1UBQ}^* - KQLEDGRTLS\text{--}\text{--}\text{--}\text{--} - 
\text{IKK2}^* - LIPDKPATQCISDGKLNEGHTLDMDLVFLFDNSKITYETQISPRQPESVSCILQEPKRN

\text{1UBQ}^* - 
\text{IKK2}^* - LAFFQLRKVWGQVWH\text{--}\text{QLKEDCNRLQQG\text{RAAMMNLLRNNSCLSKMKMSASMSQQ}\text{L}}

IKK1

\text{1UBQ}^* - MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIP\text{--}\text{--}\text{--}
\text{IKK1}^* - FVLMDHILNLIVHILNMTSAKISFLLPPDESLSLQSRIERETGINTGSQELLSETGI

\text{1UBQ}^* - PDQ\text{--} - QRLIFAG\text{--}\text{--}\text{--}\text{--} - KQLEDG\text{--} - 
\text{IKK1}^* - SLPDKPASQCVLGVRGCDSYMVLYLFDKSKTYYEGP\text{FAKSLDSVC}N\text{VYIVQD}SKIQ\text{LP}\text{I}

\text{1UBQ}^* - LRLR\text{--} - GG\text{--}\text{--}\text{--}\text{--}\text{--}\text{--}\text{--}\text{--}
\text{IKK1}^* - IQLRK\text{VAEAVHY}VGYL\text{KEDYSRLFQGQR}\text{AAMLSSLLRNANLTKMKNTLISASQQ}\text{LAKL}

}\text{**}\text{**}
F. cDNA Sources

The template DNA for subcloning NEMO and IKK2 into pET and pFast-Bac plasmids was obtained from David Rothwarf, and the Karin Lab manager respectively are shown in Figure F.1. Other plasmids include IKK1 in pET vectors constructed by Dr. Yong Qing Chen in Gouri Ghosh’s lab in early in 1998. Expression experiments to produce soluble protein were performed with IKK1 in pET plasmids, but they were not used as cloning sources in this work.
Figure F.1: DNA sources used for PCR subcloning A) Annotation of the modified pRc-β Actin plasmid which is the source of IKKα and IKKβ. B)pBK-CMV phagemid background harboring IKKγ.
G. Expression of the Bovine NEMO Zinc Finger Domain

A group from the University of Bern in Switzerland has cloned and characterized the bovine IKK proteins [98]. Crystallographers can benefit from the chemical differences found among orthologs. The currently known orthologous proteins are greater than 95% identical among vertebrates. The chemical differences may be great however, when over expressed, purified, and concentrated.

I sought to express the C-terminal zinc finger from bovine IKKγ, along with human IKKγ as a proof of concept for using IKK orthologs for future crystallographic studies. Rottenberg et al. had obtained backpac clones from a facility at the Children’s Hospital of Oakland Research Institute (CHORI), and in their paper they kindly named the bacpac clones which harbored the IKK components. I received the same material from CHORI and successfully subcloned the IKKγ subunit. Jun Cho, an undergraduate working with our lab at the time, observed that Dr. Rashmi Talwar had an N-terminal primer which we could use along with our own C-terminal IKKγ primer to PCR amplify the zinc finger DNA. The N-terminal primer introduced a silent mutation into the bovine DNA sequence. We succeeded in obtaining product from both the human template DNA and the DNA prepared from the bacteria harboring the bovine gene. We subsequently prepared pET-11a (untagged) and pET-15b (N-terminal His₆ tag) expression plasmids with these fragments, and demonstrated over expression of both the bovine and human genes.
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