Title
NMR studies of the transcriptional inhibitor I kappa B alpha and its interaction with the transcription factor NF kappa B

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

NMR Studies of the transcriptional Inhibitor I kappa B alpha and its interaction with the transcription factor NF kappa B

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

in

Chemistry

by

Carla F. Cervantes

Committee in charge:

Professor Elizabeth A. Komives, Chair
Professor H. Jane Dyson
Professor Alexander Hoffman
Professor Michael Karin
Professor Susan Taylor
Professor Peter Wolynes

2010
The dissertation of Carla F. Cervantes is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Chair

University of California, San Diego

2010
DEDICATION

“And as I look down the path I have come from, and see who I was, I see the dust of a distant past. I see the hand of the One that holds me through it all.”

Debbie Ebert, friend and writer extraordinaire

This thesis is dedicated to my family and friends who have been my faithful companions as I’ve traveled down the rabbit hole into the world science. First and foremost, to my mother, who taught me that in this short journey through life we only get to do a few things so we must be ever present and do them excellently, with her own unwavering example of being not “good” but “best” each and every time in everything she does. Thank you for being strong, independent, kind and generous. I hope to live up to your stalwart wordless example. To my family, for their unconditional love and support and for believing I could reach the stars when I could barely believe in myself. To my younger family members, thank you for making it easier for me to want to be my best every day, if only to try to create a better world for you. Friends, for your loyal support and encouragement, I am ever grateful. You have all stood by my side from year to year and inspired me in silent ways that resound in endless echoes. All of you have held up my arms during the battle when I became too tired to go on. Thus, this work is yours as much as mine because “nothing, no matter how virtuous can ever be accomplished alone” and this thesis is solid proof of it.
“Only those who will risk going too far can possibly find out how far one can go.”

*T.S. Elliot.*
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>IkBα</td>
<td>Inhibitor of kappaB alpha</td>
</tr>
<tr>
<td>IkBβ</td>
<td>Inhibitor of kappaB beta</td>
</tr>
<tr>
<td>IkBε</td>
<td>Inhibitor of kappaB epsilon</td>
</tr>
<tr>
<td>IkBδ</td>
<td>Inhibitor of kappaB delta</td>
</tr>
<tr>
<td>IKKα</td>
<td>Inhibitor of kappaB kinase α (synonymous with IKK1)</td>
</tr>
<tr>
<td>IKKβ</td>
<td>Inhibitor of kappaB kinase β (synonymous with IKK2)</td>
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<tr>
<td>IKK1</td>
<td>Inhibitor of kappaB kinase 1 (synonymous with IKKα)</td>
</tr>
<tr>
<td>IKK2</td>
<td>Inhibitor of kappaB kinase 2 (synonymous with IKKβ)</td>
</tr>
<tr>
<td>IKKK</td>
<td>Inhibitor of kappaB kinase (IKK) kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappaB</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>RDC</td>
<td>Residual dipolar couplings</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
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<tr>
<td>NOE</td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td>YLTA</td>
<td>Y254LT257A</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>hNOE</td>
<td>$^{1}$H-$^{15}$N heteronuclear NOE</td>
</tr>
<tr>
<td>R1</td>
<td>Longitudinal relaxation rate</td>
</tr>
<tr>
<td>R2</td>
<td>Transverse relaxation rate</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>HD</td>
<td>Hydrogen deuterium</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Dissociation equilibrium constant</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>TROSY</td>
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ACKNOWLEDGEMENTS

I became an undergraduate at UCSD in September 2000 with the expectation that I would become a veterinary student after completing my degree in Biochemistry. During my second year, professor Elizabeth Komives recommended me to professor Joseph Noel at the Salk Institute and I had the blessing and joy to work in his lab as a research assistant with postdoctoral fellow Paul O’Maille who was a wonderful mentor and partner as we investigated terpene cyclases. Having discovered the joy of scientific research, as well as having missed the deadline for veterinary school applications, I applied for graduate school hoping to study the biologically-relevant motions of proteins by NMR, never expecting that I would remain at UCSD. Much has happened between now and then and there are many people who I would like to acknowledge.

I would like to express my gratitude to Professor Elizabeth Komives for all her help and support throughout the years and for the opportunities and experiences that she has made available to me, first as an undergraduate, and later, as a graduate student. The opportunities she has provided to collaborate with others within and outside the lab have been an invaluable part of my growth as a scientist and essential to the work presented here. The many long hours she spent with me in the painful process of reviewing backbone assignments and teaching me the ins and outs of scientific writing and Microsoft Office will always be remembered. Through her, I first discovered the joys of scientific research and I will never forget that.

To Dr. Reina Juarez took me under her wing and mentored me, much to my surprise, despite her terribly busy schedule and my horribly awful habit of forgetting our
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assistance when I have needed it during these tiring last months, encouragement, support, camaraderie, and outright great senses of humor.

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This work is dedicated to my family and friends, and I would like to thank them here again, as I was not riding solo through this adventure: you were the voices reminding me at every step that "through many trials, toils and snares (you) have already come, was grace that brought (you) through this far and grace will lead you on".

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VITA

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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

NMR Studies of the transcriptional Inhibitor I kappa B alpha and its interaction with the transcriptional factor NF kappa B

by

Carla F. Cervantes

Doctor of Philosophy

University of California, San Diego, 2010

Professor Elizabeth A. Komives, Chair

One of the reasons why functional proteins might be unfolded or partly folded in vivo is the relative ease and rapidity by which they can be degraded when not in complex with their biological target. Previous data has indicated that the ankyrin repeat domain of IκBα, the primary inhibitor of NF-κB, may be incompletely folded in the absence of NF-κB. Based on this premise, the initial goal of this work was to investigate the solution structure and dynamics of the ankyrin repeat domain of IκBα in its free state by NMR. In the Chapters 2 and 3, solution NMR experiments on free IκBα(67-206) protein, that allowed us to characterize the structure and dynamics of this “folded” part of the IκBα ankyrin repeat domain will be presented. A battery of NMR experiments, including chemical shifts, NOEs, amide proton exchange, backbone relaxation and residual dipolar coupling measurements were performed. In Chapter 4, the complex between IκBα and a
peptide representing the nuclear localization sequence of NF-κB is characterized by NMR. Again, a battery of NMR and binding experiments are presented that show that the NLS polypeptide (residues 289-321 of NF-κB(p65) folds upon binding to IκBα providing a mechanistic explanation of how it is sequestered by inhibitor binding. In the final chapter, the full-length ankyrin repeat domain of IκBα is characterized. The fifth and sixth ankyrin repeats show very few cross peaks in the NMR indicating that they are weakly folded and in conformational exchange. By taking advantage of NMR data obtained on the bound protein as well as on a stabilized mutant, a good number of resonance assignments could be made. These assignments facilitated the interpretation of backbone dynamics experiments that allowed us to characterize the dynamics of the weakly-folded fifth and sixth repeats of the IκBα ankyrin repeat domain. Since the function of IκBα is so intimately related to its folded state, the experiments described herein should provide not only a detailed characterization of the free form of IκBα, but also important insights into its function in vivo through characterization of its complex with NF-κB.
CHAPTER 1

Introduction
NMR Studies of Intrinsically Unstructured Proteins

Despite the prevalent view that structure determines function, it is now recognized that macromolecules contain disordered regions that are important for their function. For example, motions are required for the normal function myoglobin and many other proteins (Dyson and Wright, 2004). Protein binding sites are frequently more mobile and frustrated than the rest of the protein (Ferreiro, Hegler, Komives and Wolynes, 2007). Dunker and colleagues were the first to predict that a large portion of the protein sequences encoded by the genome would be unstructured (Romero, et al., 1998). The first experimental evidence of physiologically-relevant disordered regions in proteins involved in transcriptional activation (Radhakrishnan, et al., 1997) and cell cycle regulation (Kriwacki, Hengst, Tennant, Reed and Wright, 1996) came from the Wright and Dyson groups. The field of disordered proteins has since exploded evidenced by a number of review articles in recent years (Tompa and Fuxreiter, 2008, Hazy and Tompa, 2009, Dunker, Silman, Uversky and Sussman, 2008).

NMR remains one of the few comprehensive experimental methods to obtain information on unstructured or partly structured proteins. Accurate three-dimensional structures of unstructured proteins are generally not obtainable from crystallographic studies, because conformationally disordered molecules will generally not form crystals, and if formed may not be representative of the conformational ensemble in solution. NMR can give a great deal of (less specific but more accurate) information on the conformational ensemble. Although NMR studies of disordered proteins are difficult, modern equipment and methods have yielded high-resolution information on proteins
ranging from highly denatured conformational ensembles in high concentrations of chemical denaturant to fully folded proteins that contain significant unfolded regions.

**Why Unstructured Proteins?**

Amongst the regulatory molecules that are involved in the processes of signal transduction, transcriptional activation and transcription, are a number of proteins with unstructured or partly structured domains. At least five possible advantages of intrinsically unstructured regions can be conceived. First, the presence of an unstructured regulatory molecule, for example, the p21 cyclin-dependent kinase (CDK) inhibitor would allow interaction with a multiplicity of different target molecules, such as occurs in the binding of p21 to CDKs of the cell cycle (Kriwacki, Hengst, Tennant, Reed and Wright, 1996). Second, coupled folding and binding of an unstructured domain provide a high degree of specificity in the binding interaction, with only modest increase in affinity (since there is an entropic penalty is expected for folding upon binding) (Zor, Mayr, Dyson, Montminy and Wright, 2002). This attribute is of particular relevant for transcriptional activation and signaling networks where the activation must be readily and rapidly reversible. Third, the specificity and affinity of binding of an unstructured domain to a target is increased by the large contact surface between the molecules, as, for example, when an unstructured transcriptional activator wraps completely around the target or where two domains mutually fold when they interact (Demarest, et al., 2002, Dames, Martinez-Yamout, De Guzman, Dyson and Wright, 2002). Fourth, unstructured regions are more readily modified by post-translational modification, for example, acetylation, methylation or phosphorylation. Finally, unfolded proteins are more rapidly
and completely cleared facilitating termination of the signal once it is no longer needed. Malfunctions in this clearing mechanism have been implicated in, for example, disease processes that involve the regulatory molecule p53, which has been shown to contain a number of unstructured regions (Bell, Klein, Muller, Hansen and Buchner, 2002). This latter reason could be the most relevant to the NF-κB/IκB system, the subject of this thesis.

**The NF-κB/IκB System**

The transcription factor Nuclear Factor Kappa B (NF-κB) is critical for the regulation of more than 150 genes involved in a diversity of cellular functions in response to a myriad of different signals including UV light, inflammatory cytokines, bacterial and viral products, physical or oxidative stress and apoptotic stimuli and mitogens (Baldwin, 1996, Ghosh, May and Kopp, 1998, Gerondakis, Grossmann, Nakamura, Pohl and Grumont, 1999). The genes regulated by NF-kB fall into various functional classes such as cell growth, cell differentiation, cell adhesion, apoptosis and survival, and stress response (Pahl, 1999). Consequently, proper regulation of its activity is vital for human health and it is not surprising that misregulation of NF-κB activity has been shown to be implicated in multiple human disorders. Aberrant NF-κB activity is observed, for example, in chronic inflammatory diseases (eg. arthritis and Crohn’s disease), asthma, multiple sclerosis, muscular dystrophy, heart disease, and cancer (Tak and Firestein, 2001, Yamamoto and Gaynor, 2001). Therefore, a deeper understanding of the parameters that govern the regulation of NF-κB is fundamental for understanding of the pathogenesis of these diseases and also for the development of novel therapeutics to
combat them.

**NF-κB Signaling and Regulation.** NF-κB activity is regulated by its association with its biological inhibitors, the members of the IκB family of proteins, which bind the NF-κB target and translocate it primarily to the cytoplasm (Karin and Ben-Neriah, 2000). In response to activators of this system, such as inflammatory cytokines, the IκB kinase complex phosphorylates IκBα targeting it for ubiquitynilation and subsequent degradation by the proteasome (Hoffmann, Levchenko, Scott and Baltimore, 2002). The liberated NF-κB protein then translocates to the nucleus where it binds its cognate DNA binding site and initiate gene transcription. (Figure 1.1).

**Figure 1.1** Regulation of NF-κB transcriptional activity by IκBα. NF-κB(p65)-nuclear localization signal (NLS, pink) is sequestered in the cytoplasm of resting cells by binding to its inhibitor IκBα (blue). Upon stimulation, the IκB kinase complex phosphorylates NF-κB-bound IκB, targeting it to the proteasome for degradation. Free NF-κB translocates to the nucleus, binds its cognate DNA site and activates transcription of NF-κB-responsive genes, which include that encoding for IκBα. Free IκBα is very unstable and has a lifetime of less than 10 minutes, partly due to the weakly-folded nature of the fifth and sixth repeats.
Structure of IκBα in complex with NF-κB. The major inhibitor of NF-κB activity, IκBα, is a protein whose sequence consists of two defined regions. The N-terminus is a ∼60 residue segment termed the ‘signal response region that is phosphorylated by the IKK kinase complex (Ser 32 and Ser 36). Beginning at residue 67, there is an ankyrin repeat (AR) region encompassing ∼220 amino acids, which mediates the specific interaction with NF-κB dimers. At the C-terminus, is the PEST sequence which is rich in Pro, Glu, Ser and Thr residues. The interaction between the transcription factor NF-κB and the ankyrin repeat domain of its inhibitor, IκBα, has been elucidated by x-ray crystallography (Huxford, Huang, Malek and Ghosh, 1998, Jacobs and Harrison, 1998). As shown in the crystal structure of IκBα in complex with the p50/p65 heterodimer, the NF-κB/IκBα interaction is antiparallel with the N-terminus of IκBα near the C-terminus of NF-κB (Figure 1.2).

The surface area of the interaction is extensive, burying more than 4000Å², and all six ankyrin repeats are involved in the formation of a noncontiguous contact surface. The repeats stack against each other in a linear fashion by folding into two antiparallel α-helices connected by a short loop, followed by a β-hairpin that protrudes away from the helical stack. This nonglobular fold is stabilized by hydrophobic interactions between the helices and hydrogen bonds between the hairpin-loop regions, forming a right-handed solenoid with a continuous hydrophobic core and a large solvent accessible surface area.
**Figure 1.2** Crystal structure of IκBα (blue) in complex with the p50 (green)/p65 (red) NF-κB dimer determined by Jacobs et al., 2000. The p65 nuclear localization sequence polypeptide is pictured in pink.

*Structural Characteristics of Free IκBa.* There is no detailed structural or dynamic information on the free state of IκBα. Despite much effort, attempts to crystallize the free protein proved unsuccessful (G. Ghosh, unpublished). This suggested that there might be flexible areas in the structure of IκBα. Analysis of the amino acid sequence by a neural network technique, using the program PONDR (Predictors of Natural Disordered Regions) (Romero, *et al.*, 2001, Li, *et al.*, 1999, Dunker
and Obradovic, 2001) suggested that several regions of IκBα may be unstructured. The
results of the PONDR calculation for the construct IκBα(67-317) are shown in Figure 1.3.

![Graph showing order-disorder propensities for IκBα](Image)

**Figure 1.3** Prediction of order-disorder propensities for IκBα, according to the program PONDR.

The program predicts a greater degree of disorder in the β-hairpin loops of each
ankyrin repeat and much greater disorder on the N- and C-termini of this construct.
Furthermore, according to the PONDR calculation the 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} repeats of IκBα are
almost completely structured while the 1\textsuperscript{st}, 5\textsuperscript{th} and 6\textsuperscript{th} repeats are predicted to be largely
disordered.

Hydrogen-deuterium exchange experiments monitored by mass spectrometry
performed on this construct demonstrated that, in fact, the ankyrin repeat domain of IκBα
possessed differential solvent accessibility among its ankyrin repeats, suggesting that the
first, fifth and sixth ankyrin repeats possess significant structural flexibility (Croy Hughes, Bergqvist, Huxford, G. and A., 2004), as shown in Figure 1.4.

Figure 1.4 Incorporation of solvent deuterons into amide positions of IκBα(67-317) at 25°C and pH 7.5, monitored by mass spectrometry. The solvent accessibility of peptides obtained from peptic digests of IκBα was assessed as a percentage of amide positions exchanged after 300 seconds of deuteration. These percentages have been projected onto the three-dimensional structure of IκBα (Huxford et al., 1998) as a continuous scale from least solvent accessible (blue) to most solvent accessible (red). Red regions could therefore be regarded as relatively flexible or not well-ordered. The regions not covered by a quantifiable peptide are shown in gray.

Comparison of the solvent accessibility of the ankyrin repeat β-hairpins of IκBα (measured by hydrogen-deuterium exchange monitored by mass-spectrometry), shows that there is a marked change in the solvent accessibility of the β-hairpin regions in the fifth and sixth ankyrin repeats that cannot be explained simply by protection at the
binding interface, suggesting that these regions of the protein become more structured upon binding NF-κB (Truhlar, Torpey and Komives, 2006).

**Figure 1.5** H$^2$H exchange kinetic plots for the β-hairpin regions of the ankyrin repeats of IκBα (black) and NF-κB/IκBα (red). For each graph, the maximum of the y-axis represents the maximum number of deuterons that can be incorporated for each peptide.

However, circular dichroism of free and NF-κB-bound IκBα shows that this is not due to any changes in secondary structure of IκBα, as helical content remains the same in the free and NF-κB-bound states (Figure 6). (Croy Hughes, Bergqvist, Huxford, G. and A., 2004). Our hypothesis is that the changes in solvent accessibility that are observed when IκBα binds to NF-κB primarily reflect changes in backbone dynamics and not changes in secondary structure content. Thus, the "disorder" to "order" transition that accompanies binding of IκBα to its *in vivo* target may be best probed by experiments that probe backbone dynamics, such as NMR.
Figure 1.6 CD spectra at 25 °C of free IκBα (black), NF-κB(red), the NF-κB complex (green), and the sum of the spectra of the two proteins (green).

The Importance of Conformational Disorder for Protein Function. Significant numbers of conformationally disordered proteins have been found, particularly among the regulatory molecules that bind to nucleic acids or other proteins during transcription or signaling events. There are multiple advantages for the inclusion of intrinsically unstructured regions within a protein. First of all, this permits interaction with a multiplicity of binding partners, as occurs in the binding of IκBα to the various NF-κB dimers. For example, IκBα binds to NF-κB(p50/p65) heterodimers with the same affinity as to NF-κB(p65/p65) homodimers even though the interface does not share much
sequence identify (Bergqvist, et al., 2006). Second, the simultaneous binding and folding of an unstructured domain provides an opportunity for tight binding to these multiple targets. For the examples already cited, the binding affinity is 40 pM. Finally, the presence of an unstructured region allows rapid and complete clearing of the protein once it is no longer needed, for example, by degradation, an important mode of metabolic regulation of IκBα (Hoffmann, Levchenko, Scott and Baltimore, 2002). These attributes are particularly relevant for transcriptional activation, where it is paramount to have accurate recognition leading to transcription of the correct genes, as well as a rapidly reversible interaction to terminate transcription when it is no longer needed.

Characterizing Protein Dynamics with NMR. At present, NMR is one of the few techniques able to probe the conformational dynamics of unstructured and partly unstructured proteins, yielding accurate sequence-specific information on the members of the conformational ensemble of disordered proteins. In order to understand the role of the flexibility of IκBα for its function, we will carry out NMR relaxation experiments to investigate the backbone dynamics of IκBα free and in complex with NF-κB. Furthermore, we will probe the relevance of these motions for the stability and function of IκBα by site-directed mutagenesis.
References


CHAPTER 2

Backbone resonance assignments and solution structure of the structured ankyrin repeats of IκBα
Introduction

The ankyrin repeat (AR) is a common motif in proteins that primarily function in protein-protein interactions. The AR as a recognition motif is found in more than 3500 proteins involved in numerous fundamental physiological processes across all kingdoms of life (Li, et al., 2006). For example, ARs form a scaffold for specific, high affinity interactions involved in the formation of transcription complexes, initiation of immune-responses, biogenesis and assembly of cation channels in the membranes, regulation of some cell cycle stages, and symbiotic interactions, to name a few (Sedgwick and Smerdon, 1999, Hryniewicz-Jankowska, et al., 2002). Mutations in genes encoding AR proteins can cause defects in gene expression leading to the onset and progression of disease in animals and humans (Kumar, et al., 2004). Furthermore, in recent times, there has been a significant amount of attention paid to these proteins as targets for pharmaceutical and biotechnological applications, which have been successful in several cases (Binz, et al., 2004, Schweizer, et al., 2007, Zahnd, et al., 2007).

ARs consist of 33 amino acid residues that organize into a helix-loop-helix-β-turn/loop fold. The helices are arranged in an anti-parallel fashion followed by a loop region protruding outward at an angle of ~90°, which forms a β-hairpin in known AR protein structures (Li, et al., 2006, Sedgwick and Smerdon, 1999, Mosavi, et al., 2004). In nature, ankyrin-repeat structures are found containing 1 to 33 repeats in which the helices of consecutive ARs stack together in a modular-fashion, forming a cupped-hand-shaped structure in which the helices can be visualized as the palm and the loops as the fingers (Li, et al., 2006, Sedgwick and Smerdon, 1999, Mosavi, et al., 2004). The overall
structure of this stack of ARs tends to curve slightly inward, particularly as the number of repeats increases (Li, et al., 2006, Sedgwick and Smerdon, 1999, Mosavi, et al., 2004).

IκBα is an AR protein inhibitor of the transcription factor NF-κB, which functions by sequestering NF-κB into the cytoplasm in resting state cells (Baeuerle and Baltimore, 1998). The crystal structure of IκBα in complex with NF-κB (p50/p65) shows that IκBα contacts NF-κB via its six AR domain, forming a discontinuous binding surface in which the first two ARs of IκBα contact the NLS of the p65 subunit of NF-κB and ARs 4-6 contact the dimerization domain of NF-κB p50 and p65 (Huxford, et al., 1998, Jacobs and Harrison, 1998). Until recently, there was little structural/dynamical information on free IκBα. The resistance of free IκBα to being crystallized suggested conformational disorder in the free protein. ANS binding and ¹H/²H exchange experiments monitored by mass spectrometry revealed that free IκBα possesses regions with molten globule character (Croy, et al., 2004). ¹H/²H exchange experiments further revealed that the ARs of IκBα have different solvent accessibilities, with repeats 1, 5 an 6 being the most solvent accessible (Croy, et al., 2004). Urea denaturation experiments demonstrated that the first four repeats of IκBα fold in a cooperative manner, but the fifth and sixth repeats undergo a non-cooperative folding transition (Ferreiro, et al., 2007). Furthermore, ARs 5 and 6 undergo a coupled folding and binding interaction with NF-κB whereas the first four repeats exhibit little change in solvent accessibility upon NF-κB binding (Truhlar, et al., 2006).

In order to probe the solution dynamics of the free IκBα folded ankyrin repeats, it was first necessary to obtain sequence-specific resonance assignments of the intact
molecule. Like other ankyrin repeat proteins, IκBα (67-287) is sparsely soluble and has a tendency towards aggregation in solution, particularly with rising temperature and concentration. In order to increase the “NMR lifetime” (or time before the protein aggregates and/or denatures) of IκBα(67-287) samples, it is necessary to collect data at lower temperatures (15 - 20 ºC) and concentrations (0.2 mM). Additionally, it was found that addition of 50 mM arginine and 50 mM glutamic acid prolonged the lifetime of the samples (Golovanov, et al., 2004). These conditions are less than ideal for NMR experimentation, since there is a decrease in signal at lower concentrations and line-widths tend to increase at lower temperatures. However, these are not insurmountable difficulties. Adequate data sets can be obtained under these conditions by running longer experiments in higher field instruments (800/900 MHz) to which there is access. Furthermore, the success of the NMR studies of other ankyrin repeat proteins indicate that the necessity to run experiments at lower concentrations and temperatures are not an obstacle for the NMR study of IκBα. For instance, for the INK4 proteins and gankyrin, NMR structures were elucidated at sample concentrations below 1 mM. The structures of p16INK4 and p15INK4B, the most conformationally flexible of the INK4 proteins, were solved using sample concentrations of 0.2-0.4 mM at 20 ºC. (Byeon, et al., 1998, Yuan, et al., 1999) Similarly, the structure of gankyrin, the largest and most internally homologous ankyrin repeat protein structure determined yet, was solved using samples at 0.3-0.6 mM concentration (Yuan, et al., 2004). The use of lower probe temperatures may affect coherence transfer and therefore the sensitivity of certain experiments, for example, the HNCA. However, the use of lower probe temperatures were also required
for p16<sup>INK4A</sup> and p15<sup>INK4B</sup> and adequate data sets were obtained (290K) (Byeon, et al., 1998, Yuan, et al., 1999).

**Materials and Methods**

**Expression and purification of IκBα(67-206).** IκBα(67-206) in pET11a (Novagen) was transformed into the *E.coli* BL21(DE3) strain. Expression of [<sup>2</sup>H, 15N] and [<sup>2</sup>H, 13C, 15N]-labeled IκBα(67-206) was carried out in M9 minimal media in D<sub>2</sub>O supplemented with 15NH₄Cl (2 g/L) and 13C-glucose (8g/L). Cells were acclimated by growing them sequentially in 10 mL cultures of M9ZB, M9, M9(50% D<sub>2</sub>O) and M((90% D<sub>2</sub>O). Two one-liter growths were inoculated with the M9(90% D<sub>2</sub>O) culture and induced at OD₆₀₀= 0.4 with 0.1 mM IPTG for 24 hours at 18° C. The cells were collected by centrifugation at 5000 rpm for 30 minutes and resuspended in 70 mL/liter of culture of 25 mM Tris (pH7.5), 50 mM NaCl, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 0.3 mM PMSF, and protease inhibitor cocktail(Sigma) and lysed by sonication on ice. The soluble part of the lysate was purified by cation exchange chromatography on a Hi-Load Q-Sepharose 26/10 column (GE Healthcare) using a one hour gradient from 50 to 500 mM NaCl. Final purification of the protein was done on a HiLoad Superdex 75 16/60 gel filtration column (GE Healthcare). The purified protein was concentrated in 4mL 10K MWCO Amicon concentrators (Millipore) in a fixed angle rotor at 4000 rpm for 15 minute intervals to prevent aggregation.

**NMR backbone resonance experiments.** Backbone resonance assignment experiments for IκBα(67-206) were carried out on 0.5 mM [<sup>2</sup>H, 15N, 13C] IκBα(67-206) in
25 mM Tris (pH7.5), 50 mM NaCl, 50 mM arginine, 50 mM glutamic acid, 5 mM CHAPS, 1 mM EDTA, 1 mM dithiothreitol (DTT), 2 mM NaN₃ in 90% H₂O and 10% D₂O. Standard HNCA, HN(CO)CA, HNCA CB, and HN(COCA)CB experiments were collected at 20°C on a Bruker DRX600 REFS. The parameters used for these experiments were the following: HNCA-data size=2048 (t3)x48(t2)x96(t1) complex points, number of scans=8; HN(CO)CA-data size=1024(t3)x32(t2)x90(t1) complex points, number of scans=16; HN(CA)CB, data size=1024(t3)x32(t2)x90(t1) complex points, number of scans=32; HN(COCA)CB, data size=1024(t3)x32(t2)x90(t1) complex points, number of scans=4. The delay time between each scan used was 1.5 s.

**Residual Dipolar Couplings.** The isotropic solution consisted of 0.2 mM ²H, ¹⁵N-IκBα(67-206) in the previously described buffer. The aligned solution was prepared by adding bacteriophage Pf1 (Asla Biotech) to ¹⁵N-IκBα(67-206) sample. The final sample contained 9:1 H₂O/D₂O as well as 0.2mM ²H, ¹⁵N-IκBα(67-206), 13.5 mg/mL Pf1 phage, 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM arginine, 50 mM glutamic acid, 1 mM EDTA, 2 mM NaN₃, and 2 mM DTT. All spectra for RDC experiments were taken at 20°C.

Experiments were collected using Watergate for water suppression. Residual dipolar couplings were extracted from 2D IPAP ¹H-¹⁵N HSQC spectra(Ottiger, et al., 1998). Spectra were processed with NMRpipe (Delaglio, et al., 1995) and analyzed using NMRView (Johnson, 2004). In total, 102 experimental N-H RDCs were obtained.
Results

**Backbone resonance assignments.** IκBα(67-287), which contains all six of the ARs is marginally stable (Croy, et al., 2004). In contrast, truncation of the domain at residue 206 at the end of the fourth repeat, resulted in a protein with increased solubility and much less tendency toward aggregation (Ferreiro, et al., 2007). The HSQC spectrum of IκBα(67-206) shows 133 of the 134 resonances expected (except S159), all of which have been assigned using conventional 3D HNCA, HN(CO)CA, HNCACB and HN(CO)CACB experiments (Figure 2.1). The HSQC spectrum for IκBα(67-206) exhibits resonances of even intensities, indicative of a well-structured protein unlike the that seen for IκBα(67-287) (Truhlar, et al., 2006). An exception is residues 69-72 at the N-terminus, which exhibit decreased intensity and broadening, perhaps due to intermediate exchange (~ms timescale) motions in this part of the protein.
Figure 2.1 The HSQC spectrum of 0.5 mM $^{15}$N-$^{13}$C lKB$\alpha$(67-206) collected on a 600MHz instrument at 293K.

The process of resonance assignment begins with quantification of peaks in the spectra using the HSQC and HNCO spectra (to distinguish overlapped peaks). After this, chemical shifts for all peaks in the HNCO, HNCA, and HNCACB spectra are recorded and compared to published random coil chemical shifts in order to predict the possible amino acid assignment of the residue. Next the HNCA and HNCACB spectra, (along with CBCA(CO)NH spectra to distinguish i and i-1 peaks conclusively) are used to distinguish connectivities and assign the backbone resonances. Figure 2.2 shows strip
plots for the HNCA and HNCB and HN(CO)CACB spectra for residues 101-107 (HNCO) and 173-183 (HNCA, HNCB, and HN(C)OCACB).

**Figure 2.2** Strip plots representative of the resonance assignment process, collected as described in the materials and methods section. (A) Strip plot from the HNCO spectrum, showing overlapped and non-overlapped peaks for residues 101-107. (B) Strip plots from CBCA(CO)NH spectrum (red) and HNCA spectra (black) showing connectivities for residues 99-106. (C) Strip plots from CBCA(CO)NH (red only) and HNCACB (red and black) spectra showing connectivities for residues 100-106.
The difficulty in assigning backbone resonances in the spectra of IkBα(67-206) was directly correlated to the position of the amino acid within the ankyrin repeat structure. Amino acids located in the variable loops were located in the more disperse part of the spectra and gave sharp signals making the process of assigning these resonances more straightforward. Amino acids located in the helical regions of the spectra were subject to spectral overlap and lower signal intensity than the resonances for amino acids located at the variable loops, which complicated the spectral assignment process in comparison. Figure 2.3 shows HNCA strips for residues 101-107 and 171-177 (located in the variable loops) and residues 115-121 and 184-190 (located in the inner helices) in order to illustrate this point. While the spectra for residues located in the variable loops is unperturbed by spectral overlap, which makes the process of backbone assignments straightforward (Figure 5.2.3A and B). Meanwhile, due to similar helical content and sequence similarity, residues located in the helical region are more subject to spectral overlap, which complicated the resonance assignment problem in these areas. This is similar to what is seen for other ankyrin repeat proteins which have been assigned previously where residues that comprise the helical loops are located in more disperse areas of the HSQC spectrum, while residues comprising the helices are located in the more crowded areas located in the middle of the spectra (Byeon, et al., 1998, Yuan, et al., 1999).
Figure 2.3 Strip plots from the 600 MHZ HNCA spectra of $^{15}$N-$^{13}$C-IκBα(67-206) at 293K for residues located in the variable loop regions, (A) and (B) and inner helix regions, (C) and (D).
Secondary structure of IκBα(67-206) in solution. The chemical shift differences between $^{13}$C, $^{13}$C, $^{13}$CO, $^{15}$N and HN chemical shifts for IκBα(67-206) and the sequence-corrected random coil chemical shift values are shown in Figure 2.4. An additional correction for deuterium isotope effects was also applied (Schwarzinger, et al., 2001, Gardner, et al., 1997). The $^{13}$C, and $^{13}$CO secondary chemical shifts show positive values, indicative of α-helical structure in areas that correspond well with the helical regions observed in the crystal structure. In addition, the $^{15}$C, secondary chemical shifts show the characteristic upfield/near random coil values shifts also indicating helical structure. Although the $^{1}$HN and $^{15}$N chemical shifts are a much weaker indicator of secondary structure in general, in IκBα(67–206) they show the expected upfield shifts for α-helices in areas corresponding to helical regions as well. Thus, the chemical shift measurements all indicate α-helical structure in the areas corresponding to α-helical structure observed in the X-ray crystal structure (Jacobs and Harrison, 1998).
Figure 2.4 Secondary structure of free IκBα(67-206) in solution evaluated from the chemical shift indexes of Cα, Cβ, CO, 15N and HN. Chemical shift index values are plotted versus residue number for IκBα(67-206). The chemical shift values were obtained for a sample of free [13C, 15N]-labeled IκBα(67-206) and chemical shift index values were calculated from the difference between the experimental Cα, Cβ, CO, 15N and HN chemical shifts and the corresponding random coil values. The values of ΔCα, ΔCβ, ΔCO, Δ15N and ΔHN for each residue represents the average of three consecutive residues, centered at the particular residue. Secondary structure elements determined from the crystal structure of the IκBα/NF-κB complex are shown schematically at the top for comparison.
**Residual Dipolar Couplings in IκBα(67-206).** Residual dipolar couplings (RDCs) are determined in partially-aligned media, and give direct information on the orientation of bond vectors relative to the molecular alignment tensor. RDCs are thus extremely sensitive indicators of the relative orientations of domains or structural elements within a protein (Bax, et al., 2001, Prestegard, et al., 2004) and can provide a powerful means to characterize and refine structures, particularly of helical proteins. Helix orientation in crystallographic studies can often be complicated by distortions of the conformation due to crystal packing forces or by crystallization into non-physiologically relevant configurations (Tolman and Ruan, 2006). We pursued RDC measurements to ascertain the relative orientations of the structural elements within IκBα without the need for full-scale NOE measurements (Prestegard, et al., 2004, Tolman, 2001). In order to compare the well-structured part of free IκBα in solution to the crystal structure of the IκBα•NF-κB complex, $^1$H-$^{15}$N RDCs of $^2$H, $^{15}$N-IκBα(67-206) were measured in an orienting medium containing the filamentous bacteriophage Pf1. The measured RDCs showed a periodicity consistent with the repeat structure of the AR domain; all of the helical regions showed RDC values between -5 and -10, whereas the variable loops and β-hairpins showed large, mainly positive values (Figure 2.5).
Figure 2.5 Experimentally measured $^1$H-$^{15}$N residual dipolar couplings of $\text{IkB}\alpha$(67-206) plotted as a function of residue number. Secondary structure elements determined from the crystal structure of the $\text{IkB}\alpha$/NF-$\kappa$B complex are shown schematically at the top for comparison. Helices show consecutive negative RDC values of similar magnitudes, as expected for straight structural arrays, such as $\alpha$-helices where N–H bonds are aligned parallel with the helix axis and retain the same orientation with respect to the reference frame. The similar ranges of RDC values for individual helices suggests that the $\alpha$-helices of free $\text{IkB}\alpha$(67-206) in solution are oriented similarly with respect to the alignment tensor.

The data were analyzed using the PALES software (Zweckstetter, 2008) to compare with the crystal structure of $\text{IkB}\alpha$ determined in complex with NF-$\kappa$B published by Harrison and co-workers (Jacobs and Harrison, 1998): this structure was used because it was better resolved in the region of the first two ARs of $\text{IkB}\alpha$ and contained more of the NLS sequence of NF-$\kappa$B(p65). Figure 2.4 compares experimentally-derived RDC values to those calculated by the software PALES or using an in house singular value decomposition (SVD) algorithm for the crystal structure of NF-$\kappa$B-bound $\text{IkB}\alpha$. The results showed a surprising amount of scatter and at this point it was unknown to us whether this was due to departure from the structure seen in the crystal structure of the complex or conformational exchange. This will be explored in the next chapter.
Figure 2.6 Plot of observed vs. theoretical residual dipolar couplings measured by the program PALES for \( \text{IkB\alpha}(67-206) \) (closed symbols) and SVD (open symbols) using the crystal structure of the \( \text{IkB\alpha}/\text{NF-\kappaB} \) complex (PDB accession code 1IKN, (Huxford, et al., 1998)).

Discussion

**Overall, \( \text{IkB\alpha}(67-206) \) is a well-structured AR domain.** Although many AR domains have been crystallized, the AR domain of \( \text{IkB\alpha} \) has remained resistant to crystallization. Earlier work using native-state \( \text{H}^2 \text{H} \) exchange experiments at short time intervals showed that the fifth and sixth repeats were fully exchanged after only two min despite the fact that all of the helical secondary structure seemed to be present (Croy, et al., 2004, Ferreiro, et al., 2007). Many attempts at truncation of \( \text{IkB\alpha} \) to produce a well-behaved and well-structured fragment failed, but truncation at residue 206 resulted in a
soluble protein with better solubility properties than the full-length AR domain. The NMR results presented here strongly indicate that this fragment of IκBα is, in fact, well-folded and therefore the "instability" of the full-length AR domain can be attributed to ARs 5 and 6, but not to ARs 1-4. Heteronuclear NOEs and amide H²H exchange protection factors show that this part of the protein behaves as a well-structured AR domain similar to others for which structures and dynamics have been obtained including p16\textsuperscript{INK4A}, p18\textsuperscript{INK4C} and p19\textsuperscript{INK4D}.

Since the structure of this AR domain was already determined by x-ray crystallography, albeit in complex with its binding partner, NF-κB, we elected not to solve the structure again. Instead, we performed RDC experiments to ascertain whether there were any differences between the structures of the AR domain before and after binding. Careful analysis of the RDC data revealed that the data from a single helix, the outer (helix 2) of AR3, differed in the free and bound forms.

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*These authors contributed equally to this work.
References


Chapter 3

Functional dynamics of the structured ankyrin repeats of IκBα
Introduction

The ankyrin repeat (AR) is a common motif in proteins that function primarily in protein-protein interactions. The AR is found as a recognition motif in more than 3500 proteins involved in numerous fundamental physiological processes across all kingdoms of life (Li, et al., 2006). For example, ARs form a scaffold for specific, high affinity interactions involved in the formation of transcription complexes, initiation of immune responses, biogenesis and assembly of cation channels in membranes, regulation of some cell cycle stages, and symbiotic interactions (Sedgwick and Smerdon, 1999, Hryniewicz-Jankowska, et al., 2002). Mutations in genes encoding AR proteins can cause defects in gene expression leading to the onset and progression of disease in animals and humans (Kumar, et al., 2004). Furthermore, these proteins have recently been successfully targeted in pharmaceutical and biotechnological applications (Binz, et al., 2004, Schweizer, et al., 2007, Zahnd, et al., 2007).

Most naturally occurring ankyrin-repeat domains contain multiple repeats that form a cup-shaped structure in which the helices form the convex surface and the β-hairpins protrude into the concave surface (Li, et al., 2006, Sedgwick and Smerdon, 1999, Mosavi, et al., 2004). Each AR consists of 33 amino acids organized into a β-turn/loop-helix-loop-helix fold. The β-hairpin and loop region protrude outward at an angle of ~90° with respect to the helices, which are arranged in an anti-parallel fashion. To date, four structures have been solved by NMR (Yuan, et al., 1999, Li, et al., 1999, Luh, et al., 1997, Yang, et al., 1998) and some fifteen more are available from crystallography. Both the convex and concave surfaces of AR domains are used to mediate protein-protein
interactions, with the concave surface of the b-hairpins forming the binding interface in the majority of complexes studied so far. Structures of AR domains in complex with their binding partners have also been solved both by NMR (Yang, et al., 2009) and crystallography.

The AR domain-containing protein, IκBα, is an inhibitor of the transcription factor NF-κB, and functions by sequestering NF-κB in the cytoplasm in resting-state cells (Baeuerle and Baltimore, 1998). The crystal structure of IκBα in complex with NF-κB (p50/p65) shows that IκBα contacts NF-κB via its six AR domains, forming a discontinuous binding surface in which the first two ARs of IκBα contact the NLS of the p65 subunit of NF-κB and ARs 4-6 contact the dimerization domain of NF-κB p50 and p65 (Huxford, et al., 1998, Jacobs and Harrison, 1998). Until recently, there was little structural or dynamic information on free IκBα. The resistance of free IκBα to crystallization (G. Ghosh, personal communication) suggests the presence of conformational disorder in the free protein. ANS binding and H/D exchange experiments monitored by mass spectrometry revealed that free IκBα possesses regions with molten globule character (Croy, et al., 2004). H/D exchange experiments further revealed that the ARs of IκBα have different solvent accessibilities, with repeats 1, 5 and 6 being the most solvent accessible (Croy, et al., 2004). Urea denaturation experiments demonstrated that the first four repeats of IκBα fold in a cooperative manner, but the fifth and sixth repeats undergo a non-cooperative folding transition (Ferreiro, et al., 2007). Furthermore, ARs 5 and 6 undergo a coupled folding and binding interaction with NF-
κB, whereas the first four repeats exhibit little change in solvent accessibility upon NF-κB binding (Truhlar, et al., 2006).

In order to understand the architecture and dynamics that hold the well-structured core of free IkBα together, we have undertaken NMR experiments on IkBα(67-206), which comprises the first four ARs of the protein. The combined results of NMR dynamics, H/D exchange, and RDC experiments reveal many structural similarities to the crystal structure of NF-κB-bound IkBα, and some surprising differences that were suggested in previously-published all-atom molecular dynamics simulation of IkBα(67-287) (Ferreiro, et al., 2007). As suggested by previous H/D exchange mass spectrometry experiments, IkBα(67-206) is well-structured, but backbone 15N order parameters reveal fast time-scale dynamics in the variable loops and microsecond-millisecond dynamics in the β-hairpins. Furthermore, RDC analysis combined with the results from NMR relaxation and 1H/2H exchange experiments show that, while the orientations of the eight helices of this domain are very similar to the orientations seen in the crystal structures (Huxford, et al., 1998, Jacobs and Harrison, 1998), the outer helix of AR3 is undergoing dynamics which may have implications for the function of IkBα.

Materials and Methods

Expression and purification of IkBα(67-206). IkBα(67-206) in pET11a (Novagen) was transformed into the E.coli BL21(DE3) strain. Expression of [²H, ¹⁵N] and [²H, ¹³C, ¹⁵N]-labeled IkBα(67-206) was carried out in M9 minimal media in D²O supplemented with ¹⁵NH₄Cl (2 g/L) and ¹³C-glucose (8g/L). Cells were acclimated by
growing them sequentially in 10 mL cultures of M9ZB, M9, M9(50% D$_2$O) and M9 (90% D$_2$O). Two one-liter growths were inoculated with the M9(90% D$_2$O) culture and induced at OD$_{600}$= 0.4 with 0.1 mM IPTG for 24 hours at 18°C. The cells were collected by centrifugation at 5000 rpm for 30 minutes and resuspended in 70 mL/liter of culture of 25 mM Tris (pH7.5), 50 mM NaCl, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 0.3 mM PMSF, and protease inhibitor cocktail (Sigma) and lysed by sonication on ice. The soluble part of the lysate was purified by cation exchange chromatography on a Hi-Load Q-Sepharose 26/10 column (GE Healthcare) using a one hour gradient from 50 to 500 mM NaCl. Final purification of the protein was done on a HiLoad Superdex 75 16/60 gel filtration column (GE Healthcare). The purified protein was concentrated in 4mL 10K MWCO Amicon concentrators (Millipore) in a fixed angle rotor at 4000 rpm for 15 minute intervals to prevent aggregation.

**NMR Relaxation Measurements.** T$_1$, T$_2$, and [${}^1$H]-{}^{15}$N$ heteronuclear NOE measurements for 0.5 mM 2H-$^{15}$N IKBa(67-206) were carried out at 20°C on Bruker Avance 501 and DRX600 using standard Bruker programs. T$_1$ delays were 12(duplicate), 177, 353, 705, 1057(duplicate), 1409, 1761, 2201, and 2817(duplicate) ms. T$_2$ delays were 9(duplicate), 13, 17 21, 29 (duplicate), 37 53, and 61 (duplicate) ms. [${}^1$H]-{}^{15}$N$ NOE saturated and unsaturated spectra measurements were recorded in an interleaved manner. Data were processed using NMRpipe (Delaglio, et al., 1995) and analyzed using NMRView (Johnson, 2004) and Curvefit (Mandel, et al., 1995).

**H/D Exchange experiments.** The amide H/D exchange was measured on, 2H, $^{15}$N-labeled IKBa(67-206) by rapidly exchanging the protein from H$_2$O buffer to D$_2$O (pD=7.1) buffer using a desalting spin column on ice. The sample was immediately put
into the NMR tube and into the spectrometer. Progress of the exchange of the amide protons with deuterium was followed by collecting a series of successive \(^1\)H, \(^{15}\)N-HSQC spectra starting immediately after the buffer exchange into D\(_2\)O buffer. All exchange experiments were conducted on a Bruker DRX600 at 20 °C, for a total exchange time of 30 hr. The first HSQC spectrum was collected after 15 min, and the rest of the spectra were acquired at a 30 min interval for the first 4 hr. The last spectrum was acquired with the sample in the spectrometer for overnight (18 hr). Protection factors (PF) were determined by calculating \(k_{\text{int}} / k_{\text{ex}}\) where \(k_{\text{ex}}\) is the exchange rate constant obtained by fitting a single \(-\)exponential function to the intensities of amides in the series of HSQCs and \(k_{\text{int}}\) is the intrinsic exchange rate constant obtained by using the program SPHERE (Bai, et al., 1993) which corrects for pH and temperature effects. To obtain an estimate for the standard errors, each exchange experiment was repeated twice.

**Residual Dipolar Couplings.** The isotropic solution consisted of 0.2 mM \(^2\)H, \(^{15}\)N-I\(\kappa\)B\(\alpha\)(67-206) in the previously described buffer. The aligned solution was prepared by adding bacteriophage Pf\(\phi\) (Asla Biotech) to \(^{15}\)N-I\(\kappa\)B\(\alpha\)(67-206) sample. The final sample contained 9:1 H\(_2\)O/D\(_2\)O as well as 0.2mM \(^2\)H, \(^{15}\)N-I\(\kappa\)B\(\alpha\)(67-206), 13.5 mg/mL Pf\(\phi\) phage, 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM arginine, 50 mM glutamic acid, 1 mM EDTA, 2 mM NaN\(_3\), and 2 mM DTT. All spectra for RDC experiments were taken at 20° C.

Experiments were collected using Watergate for water suppression. Residual dipolar couplings were extracted from 2D IPAP \(^1\)H-\(^{15}\)N HSQC spectra(Ottiger, et al., 1998). Spectra were processed with NMRpipe (Delaglio, et al., 1995) and analyzed using NMRView (Johnson, 2004). In total, 102 experimental N-H RDCs were obtained.
**Accelerated Molecular Dynamics.** The details of the accelerated molecular dynamics (AMD) method have been discussed previously in the literature (Hamelberg, et al., 2004, Hamelberg and McCammon, 2005). In AMD, a continuous non-negative bias potential is added to the original potential energy surface. This results in a raising and flattening of the potential energy landscape, decreasing the magnitude of the energy barriers between low energy states, and therefore enhancing the escape rate from one low energy conformational state to another, whilst maintaining the essential details of the underlying potential energy surface. The extent to which the potential energy surface is modified depends on the difference between the boost energy and the actual potential. Explicitly, the modified potential, \( V^*(\vec{r}) \), is defined as:

\[
V^*(\vec{r}) = \begin{cases} 
V(\vec{r}) & \text{if the potential energy, } V(\vec{r}), \text{ is equal to or greater than the boost energy, and} \\
V(\vec{r}) + \Delta V(\vec{r}) & \text{if the potential energy is less than the boost energy.}
\end{cases}
\]

The energy modification, or 'bias' is given by:

\[
\Delta V(\vec{r}) = \frac{(E_b - V(\vec{r}))^2}{\alpha + (E_b - V(\vec{r}))}
\]

The extent of acceleration (ie. how aggressively the conformational space sampling is enhanced) is determined by the choice of the boost energy, \( E_b \), and the acceleration parameter, \( \alpha \). One of the favorable characteristics of AMD is that the corrected canonical ensemble average can be back calculated, so that thermodynamic and other equilibrium properties of the system can be accurately determined.

The protocol employed in this study follows along very similar lines to a detailed study of the RDCs in the proto-typical system ubiquitin (Markwick et al., submitted). All simulations were performed using a modified in-house version of the AMBER10 code.
The coordinates for IκBα(67-206) were obtained from the X-ray crystal structure of the IκBα/NFκB complex (PDB code 1NFI). The system was placed in a periodically repeating box with 8,000 water molecules and eight Na⁺ counter-ions. For each simulation, the system was brought to thermodynamic equilibrium at 300K, 1 bar pressure using a Langevin thermostat with a collision frequency of 3-ps⁻¹ and a Berendsen weak-coupling pressure-stat. Simulations were performed for 10-ns each under periodic boundary conditions with a time-step of 1-fs. Electrostatic interactions were treated using the Particle Mesh Ewald method (PME) (Cheatham, et al., 1995) with a direct space sum limit of 10-Å. The ff99SB force-field (Hornak, et al., 2006) was used for the solute residues and the TIP4P water force-field was employed for the solvent molecules. A series of five standard MD simulations acted as a control set, were used as the starting point for the AMD simulations and also provided an estimate of the average dihedral angle energy (Vdih). For IκBα a “dual boost” AMD methodology (Hamelberg, et al., 2007) was used, whereby, in addition to the acceleration that is applied across the torsional terms of the force-field, a fixed background acceleration was also applied across the entire potential. This background potential is weak, with acceleration parameters \( \alpha(\text{tot}) = (0.2 \times \text{total number of atoms in system kcal/mol}) \) and \( E_b(\text{tot}) - V(\text{tot}) = \alpha(\text{tot}) \). The AMD simulations were performed for 10,000,000 steps at increasing levels of torsional acceleration with otherwise identical parameters as the standard simulations. Twenty AMD simulations were performed at each acceleration level. The corrected canonical ensemble was determined by performing a free energy weighting protocol. The strict Boltzmann re-weighting criterion was relaxed and an initial free energy ‘pre-pruning’ for each AMD ensemble was performed in which the high energy structures
were stripped out (some 80% of the total trajectory) and the remaining 20% was used to perform the clustering analysis (Massova and Kollman, 1999). To obtain accurate free energy statistics, a reduced set of structures that represent the conformational space sampled in the trajectory were used to seed classical MD simulations. These were then subjected to MM/PBSA analysis which gives the relative free energies. In this way, the AMD simulations were primarily employed to obtain enhanced conformational space sampling, whilst the free energy statistics are provided by the MM/PBSA analysis. The ‘optimal’ torsional acceleration level for the best reproduction of the experimental RDCs was found to be Eb(dih) − V(dih) = 600 kcal/mol, α(dih)=120 kcal/mol. A detailed description of the results of the entire simulation study will be provided elsewhere (Markwick et al., submitted).

An SVD analysis was performed to determine the optimal alignment tensor for molecular ensembles generated at each acceleration level (Showalter and Brüschweiler, 2007). RDCs calculated from each ensemble at a given acceleration level were then averaged to include the effect of statistical mechanical sampling, as no single free-energy weighted trajectory generated from an AMD simulation reproduced the experimental data as well as the trajectory average. Consistent with our findings for ubiquitin, neither the single X-ray crystal structure, nor a set of ensembles generated from standard MD simulations yielded calculated RDCs that fit the experimental data as well as the trajectory averaged optimal AMD result (Markwick et al. (submitted). Trajectory-averaged order parameters (the orientational distribution of each NH bond vector) were also determined at the RDC-optimal acceleration level.
**Residual Dipolar Couplings.** We first used the program PALES and an in house SVD algorithm to find the best alignment tensor for the experimental RDC data (using all 102 RDCs and the structure from PDB accession number 1NFI) (Jacobs and Harrison, 1998, Zweckstetter, 2008). The SVD analysis revealed that only 97 of the 102 experimental RDCs could be fit. RDCs for residues 75 and 77, found in the highly flexible N-terminal tail and for residue 81, which had a non-uniform peak shape, were ignored. Two other RDCs, for residues 118 and 184 were removed from the analysis because their values were found to lie significantly outside the alignment tensor window. These five RDCs also did not fit those generated from the X-ray crystal structure and from the MD generated molecular ensembles. Using the criterion of 1Hz as significance, the experimental RDCs for three residues; 96, 199 and 200, fit better to the crystal structure than to the AMD molecular ensemble. Remarkably, the experimental RDCs for 40 residues showed a significant improvement in the fits to the AMD ensemble as compared to the crystal structure. Of these, 29 (bold face) also showed long timescale dynamics (residues 77, 80, 86, 87, 88, 89, 98, 99, 100, 101, 104, 105, 106, 120, 125, 127, 128, 130, 131, 133, 135, 136, 141, 144, 145, 146, 156, 158, 164, 167, 168, 173, 177, 180, 186, 189, 193, 194, 201, and 206). The improvement in the fit of the other 11 RDCs is most likely due to an improved representation of the time- and ensemble-averaged alignment tensor. Slow collective dynamic motions may alter the shape anisotropy of the system, particularly for repeat proteins. The N-H bond vectors associated with these residues happen to lie at a critical angle relative to the alignment tensor, where even small variations in the orientation of the alignment tensor produce substantial changes in the
corresponding RDC. A good example of this is residues 120 and 131, which show improvements of 3.37 Hz and 5.14 Hz respectively.

**Results**

**Backbone Dynamics of IκBα (67-206).** In order to probe the ps-ns scale motions for IκBα(67-206) we measured $^{15}$N-R$_1$, $^{15}$N-R$_2$ and $[^{1}H]^{15}$N heteronuclear NOE values at 500 and 600 MHz (Figure 3.1). The majority of the heteronuclear NOE values were 0.8 or greater, as expected of a well-structured protein. However, residues 68, 71, and 77 near the N-terminus, residues 99 and 100 in the variable loop between AR1 and AR2, and residue 206 at the C-terminus, showed values less than 0.6, indicative of flexibility in the ps-ns timescale.
Figure 3.1. Backbone relaxation data ((A) $R_1$, (B) $R_2$, and (C) heteronuclear NOE) were collected for $[^1H, ^{15}N]$ IκBα(67-206) at 500 and 600 MHz as described in the Methods section. (D) H/D exchange results are plotted for comparison. Secondary structure elements determined from the crystal structure of the IκBα/NF-κB complex are shown schematically at the top for comparison.
TENSOR2 was used to analyze the relaxation data (Dosset, et al., 2001) according to the Model-free formalism (Lipari and Szabo, 1981). The resulting order parameters (Figures 3.2) show the presence of ps-ns time scale dynamics in the variable loops between each AR and in the β-hairpins at the beginning of each AR. Several regions exhibited longer timescale motions. The β-hairpins fit better to a model that included μs – ms motions (Figure 3B). Long-time scale motions were seen particularly for residues 73,77,86, and 88 in AR1, which comprise the majority of the contact surface between the first AR and helix 4 of NF-κB(p65) in the bound complex (Huxford, et al., 1998, Jacobs and Harrison, 1998). The latter half of the AR2 β-hairpin (residues 108-113), the entire β-hairpin of AR3 (residues 135-146), and the variable loop between AR3 and AR4 as well as the entire β-hairpin of AR4 (residues 169-185) also showed evidence of longer time-scale motions.
Figure 3.2. Modelfree parameters calculated from the $^{15}$N relaxation data of free IκBα(67-206) using the program TENSOR2. (A) The generalized order parameter ($S^2$) of NH vectors plotted as a function of residue number. (B) The apparent chemical/conformational exchange contribution ($R_{ex}$) to the transverse relaxation rate $R_2$. Residues exhibiting $R_{ex}$ are located in areas of decreased $S^2$ values. Secondary structure elements determined from the crystal structure of the IκBα:NF-κB complex are shown schematically at the top for comparison.

Hydrogen/Deuterium Exchange monitored by NMR spectroscopy. H/D exchange experiments were performed on IκBα(67-206) in order to obtain site-specific information on the local stability (and flexibility) of the backbone of IκBα(67-206) toward unfolding processes. Previous mass spectrometry-based H/D exchange experiments (Truhlar, et al., 2006) were performed only for times up to five minutes, and
little amide exchange was observed in this region of the protein. NMR H/D exchange experiments cannot access such short time scales, but give information on longer time scales from 20 min to hours. Cross-peak intensities observed in $^1$H-$^{15}$N heteronuclear quantum coherence (HSQC) spectra were monitored during a total exchange time of 30 hrs after rapid solvent exchange into D$_2$O buffer at pH 7.5 and the data fit to a single exponential decay for each cross peak. The protection factors were derived by dividing the intrinsic exchange rate constants for each amino acid (Englander, et al., 1997) by the experimentally determined rates. Protection factor values ranged from $10^4$ to $10^6$, indicative of a very well structured protein. Residues for which the exchange was too rapid to measure a protection factor included 69, 71, 74-74 (N-terminus), 97-103 (AR1/2 variable loop), 132, 134, 140, 142-143 (AR 2/3 variable loop), 156-157, 161, 165-166,(AR3 helix 2) 168-169, 174-177, 179, 179-185 (AR3/4 variable loop), 187-188(AR4 helix 1), 193-196 (loop between helix 1 and helix 2 in AR4), 205-206 (C-terminus). Higher protection factors were observed in the second AR and the first half of the third AR, consistent with the mass spectrometry-based experiments on shorter timescales for IκBα(67-287) (Truhlar, et al., 2006). The lower protection factors that we observed in the first AR of IκBα(67-206) were also seen in the mass spectrometry-based measurements, but the lower protection factors in the fourth AR are due to the absence of the fifth and sixth ARs from the shorter construct studied here.

**Residual Dipolar Couplings in IκBα(67-206).** Residual dipolar couplings (RDCs) are determined in partially-aligned media, and give direct information on the orientation of bond vectors relative to the molecular alignment tensor. RDCs are thus extremely sensitive indicators of the relative orientations of domains or structural
elements within a protein (Bax, et al., 2001, Prestegard, et al., 2004) and can provide a powerful means to characterize and refine structures, particularly of helical proteins. Helix orientation in crystallographic studies can often be complicated by distortions of the conformation due to crystal packing forces or by crystallization into non-physiologically relevant configurations (Tolman and Ruan, 2006). We pursued RDC measurements to ascertain the relative orientations of the structural elements within IκBα without the need for full-scale NOE measurements (Prestegard, et al., 2004, Tolman, 2001). In order to compare the well-structured part of free IκBα in solution to the crystal structure of the IκBα•NF-κB complex, $^{1}$H-$^{15}$N RDCs of $^{2}$H, $^{15}$N-IκBα(67-206) were measured in an orienting medium containing the filamentous bacteriophage Pf1. The measured RDCs showed a periodicity consistent with the repeat structure of the AR domain; all of the helical regions showed RDC values between -5 and -10, whereas the variable loops and β-hairpins showed large, mainly positive values (Figure 3.3A). The data were analyzed using the PALES software (Zweckstetter, 2008) to compare with the crystal structure of IκBα determined in complex with NF-κB published by Harrison and co-workers (Jacobs and Harrison, 1998): this structure was used because it was better resolved in the region of the first two ARs of IκBα and contained more of the NLS sequence of NF-κB(p65). Figure 3.3A compares experimentally-derived RDC values to those calculated by the software PALES or using an in house singular value decomposition (SVD) algorithm for the crystal structure of NF-κB-bound IκBα. The results showed a surprising amount of scatter especially considering the high order parameters of the domain. Although RDCs are a structural measure, they reflect dynamic
averaging within the structural ensemble up to a time scale of 1/D (the inverse of the coupling constant), which is typically several milliseconds (Tolman, et al., 1997).

**Accelerated molecular dynamics of IκBα(67-206).** Accelerated molecular dynamics (AMD) simulations (see Methods) were pursued to ascertain whether dynamic processes were contributing to the scatter observed in the RDCs. Unrestrained AMD simulations were carried out at increasing acceleration levels to provide systematically enhanced conformational space sampling. Several simulations were obtained for each acceleration level to generate multiple ensembles. SVD analysis of the ensembles collected at each acceleration level was performed to obtain the optimal or preferred alignment tensor producing the best possible reproduction of the experimental RDC data for each molecular ensemble. This procedure is identical to that of PALES to obtain the optimal alignment tensor for the X-ray crystal structure that best reproduces the RDC data, except that it is now being performed on ensembles of structures. It should be emphasized that in comparison to other studies concerning the structural/dynamic interpretation of RDCs, the present method is not based on a “fitting” procedure and doesn’t invoke the use of a predefined model of internal dynamics (Tolman, 2002, Meiler, et al., 2003, Bouvignies, et al., 2006, Lange, et al., 2008). Instead, the RDCs were calculated from the free-energy weighted ensembles at increasing acceleration levels. Importantly, the method used here does not require multiple sets of RDC data acquired in different alignment media. The acceleration level that yielded RDCs for the ensembles that best matched those from the experiment was identified (Figure 3.3B). Clearly, the ensembles represent the measured RDC values much better than the single structure found in the crystal. Of the 98 residues, 75 showed some improvement and 40
RDCs showed a significant improvement of at least 1 Hz. Of these, 29 were associated with residues in regions of the protein that sampled extended conformational space over slower time scales in the optimized AMD ensemble. The other 11 residues that significantly improved were not in regions where slow timescale dynamics were occurring. We attribute the significant improvement in these RDCs to the improved representation of the time-averaged ensemble alignment tensor, and recognize that many of these residues had NH bond vectors that critically depended on this parameter.

Figure 3.3. (A) Plot of observed vs. theoretical residual dipolar couplings measured by the program PALES for IκBα(67-206) (closed symbols) and SVD (open symbols) using the crystal structure of the IκBα/NF-κB complex (PDB accession code 1IKN, (Huxford, et al., 1998)). (B) Plot of observed vs. AMD-calculated residual dipolar couplings for IκBα(67-206). There is significant improvement in the correlation of the AMD-calculated RDCs (R-factor 0.97) with the observed RDCs compared to the results from PALES or SVD (R-factor 0.80). For the RDC measurements, an aligned solution was prepared by adding bacteriophage Pf1 (Asla Biotech) to a $^{15}$N-IκBα(67-206) sample. All spectra were acquired at 293K.
A representative ensemble of structures from an AMD simulation at the acceleration level that best fit the RDC data is shown in Figure 3.4. This ensemble shows structural variations particularly in the outer helices within AR1 and AR3 as well as the variable loops. The trajectory-averaged NH bond vector order parameters were calculated for the RDC-optimal acceleration level, and are plotted in Figure 6B. Experimental $^{15}\text{N}$ spin relaxation order parameters, describing dynamics on time-scales up to 6 ns are also plotted again for comparison. The AMD order parameters calculated at the RDC-optimal acceleration level are not limited by the rotation diffusion time, but report on dynamic motions up to milli-second time-scales ($1/D$, where $D$ is the magnitude of the NH RDCs). We observe a heterogeneous distribution of long time-scale dynamics across the system. Interestingly, many of the regions where the RDC-optimal order parameters are lower than those obtained from NH spin relaxation coincide with those residues that exhibit exchange relaxation (micro-millisecond dynamics). The experimental spin relaxation (Figure 3.4A) and the order parameters obtained from the best-fit AMD ensemble (Figure 3.4B) are displayed on the structure of $\text{IκBα}(67-206)$ using a temperature scale.
Figure 3.4 (A) Structure of IκBα(67-206) from the the IκBα/NF-κB complex (PDB accession code 1NFI, (Jacobs and Harrison, 1998)) showing the order parameters (S²) determined from the TENSOR2 analysis of the R₁, R₂, and hNOE data. (B) Ensemble of structures from the AMD simulation using the ‘optimal’ torsional acceleration level for the best reproduction of the experimental RDCs was found to be Eb(dih) – V(dih) = 600 kcal/mol, α(dih)=120 kcal/mol. The calculated order parameters (S²) determined from the NH bond vectors from the ensemble-weighted average are shown on the structures. The color scales for both plots are red to blue for values of S² = 0-1.

Comparison of free vs. bound IκBα. We also used Tensor2 to analyze the R₁, R₂, and heteronuclear NOE data collected previously on NF-κB-bound IκBα(67-287) (Sue, et al., 2008)and compared the results to those already presented for IκBα(67-206). Only three residues within the first four ankyrin repeats, 70, 83, and 185 required the model that included R₁, yielding values of 38.6, 9.4, and 16.5 sec⁻¹ respectively. Thus, markedly fewer residues showed slow dynamics (R₁) in the complex as compared to the
free protein (Figure 3.2B). The $R_{ex}$ values were determined from the analysis using the anisotropic model in both cases, although similar results were also obtained from the isotropic model. Order parameters for $\text{IκB}_\alpha$ bound to NF-κB compared well with those for free $\text{IκB}_\alpha$. However, the order parameters in the variable loops were generally lower for $\text{IκB}_\alpha$ in the complex as compared to free. This can be explained by the fact that the complex is larger, has a slower rotational diffusion time (13 ns compared to 6 ns) and therefore the order parameters are reporting on internal dynamics over slightly longer timescales (Figure 3.5). The fact that both isotropic and anisotropic models gave similar results, and that the rotational diffusion time is obtained from the ratio of $R_1/R_2$ initially, with no reference to the heteronuclear NOE data, suggest that the effect of the different rotation diffusion times (and models) on the internal dynamics should not be significant.

Figure 3.5. Structure of the $\text{IκB}_\alpha$NF-κB complex (PDB accession code 1NFI, (Jacobs and Harrison, 1998)) showing those residues of $\text{IκB}_\alpha$ (67-206) with significant $R_{ex}$ colored in red.
Discussion

IκBα(67-206) is a well-structured AR domain. Although many AR domains have been crystallized, the AR domain of IκBα has remained resistant to crystallization except in complex with its binding partner, NF-κB. Earlier work using native-state H/D exchange experiments at short time intervals showed that the fifth and sixth repeats were fully exchanged after only 2 min despite the fact that all of the helical secondary structure seemed to be present (Croy, et al., 2004, Ferreiro, et al., 2007). Truncation of IκBα at residue 206 resulted in a protein with better solubility properties than the full-length AR domain. The NMR results presented here strongly indicate that this fragment of IκBα represents the well-folded part of the IκBα AR domain. The overall high heteronuclear NOE values and amide H/D exchange protection factors obtained for IκBα(67-206) show that this part of the protein behaves as a well-structured AR domain similar to AR proteins which have been structurally and dynamically characterized by NMR including p16INK4A, p18INK4C and p19INK4D (Yuan, et al., 1999, Renner, et al., 1998).

Since the structure of this AR domain was already determined by x-ray crystallography, albeit in complex with its binding partner, NF-κB, we elected not to solve the solution structure of the free protein by standard NOE-based methods, but rather to perform RDC experiments to ascertain whether there were any differences between the structures of the AR domain free in solution and bound to NF-κB in the crystal. Careful analysis of the RDC data revealed differences in the outer helices (helix 2) of ARs 1 and 3. In addition, it was possible to carry out a full backbone relaxation
analysis of ARs 1-4 of IκBα, and the results reveal multiple timescales of motion centered around the binding interface in the complex with NF-κB.

**Dynamic motions in the outer helices (helix 2) of AR1 and AR3.** We recently reported NMR studies of the full AR domain of IκBα(67-287) bound to NF-κB (Sue, et al., 2008). Comparison of the cross peak intensities of free IκBα(67-206) with those of bound IκBα(67-287) revealed that many of the cross peaks in AR3 disappeared in the spectrum of the bound form. In addition, protection factors could not be measured for most of AR3 in the complex, but they are high in the free protein (Suppl Figure 2). Residues that are missing in the spectra of the IκBα(67-287)•NF-κB complex include residues 135 through 156, corresponding to the first half of AR3 (Sue, et al., 2008). In the free protein, the variable loops between AR2 and AR3, and the variable loop between AR3 and AR4 appear to have greater flexibility in the free IκBα(67-206) than in the bound. Whereas cross peaks for residues 147-156 (helix 1) were missing in the bound protein, these are well-ordered, as indicated by high S^2 values, absence of R_{ex}, and measurable protection factors in the free IκBα(67-206) (Figure 3.2). Consistent with what was observed in the bound protein, few of the amides in AR3 helix 2 exchanged slowly enough to even measure protection factors even though the cross peaks were clearly visible in the HSQC. These observations were also corroborated by the AMD simulations which showed a broader ensemble of structures for helix 2 of AR3 than for the other helices in the molecule except for helix 2 of AR1, which can be explained by the fact that it is in the first repeat.

The observation of slow timescale conformational dynamics in the AR3 outer helix of the free protein is significant for several reasons. First, the interface between the
second and third ARs of IκBα is predicted to be the site of folding nucleation, so one might expect that AR3 would not be conformationally mobile (Ferreiro, et al., 2005). Second, it is known that the interface between NF-κB and IκBα has two hot spots, one at either end (Bergqvist, et al., 2008). This led us to the hypothesis that upon binding, the AR domain of IκBα might be "squeezed". This hypothesis was supported by the observation that cross peaks in AR3 disappear upon binding hinting that the "squeezing" might cause intermediate exchange in AR3. We had previously suggested that the "squeezing" might contribute to an entropy compensation for folding of the fifth and sixth repeats. However, only small entropy changes are observed upon binding and prefolding of the fifth and sixth repeats do not result in large entropy “savings” upon binding (Truhlar, et al., 2008). The present results suggest that instead of an overall increase in flexibility of AR3 upon binding, there is a more specific entropy compensation between structural elements within AR3. It is interesting that comparison of the order parameters of residues 67-206 in the free vs. bound IκBα actually show similar and sometimes lower order parameters in the bound form (Figure 3.4). Overall, the ARD underwent slower timescale dynamics when free than when bound to NF-κB.

The binding interface has slow timescale dynamics. Whereas many residues in the free protein showed slow timescale dynamics as evidenced by the requirement of additional $R_{ex}$ terms to fit the relaxation data, only three residues showed $R_{ex}$ in the bound form. Figure 3.5 shows the residues for which longer timescale motions were detected mapped onto the structure of the complex (Jacobs and Harrison, 1998). These residues correspond very well to the surface of the first four ARs of IκBα that contacts NF-κB(p50/p65). The interaction between the ARD of IκBα and NF-κB(p50/p65) involves
coupled folding and binding; both of the fifth and sixth ARs of IκBα folding onto the dimerization domains of NF-κB (Truhlar, et al., 2006) and of the NLS of p65 folding onto the first AR of IκBα (Cervantes et al., unpublished data). Comparison of the crystal structures of NF-κB(p50/p65) bound to DNA and bound to IκBα show that whereas the C-terminal residues of p65, which contain the NLS sequence, are not structured in the DNA-bound complex, they form a bent helical segment when bound to IκBα (Jacobs and Harrison, 1998, Chen, et al., 1998). This latter folding upon binding event results in the interface shown in Figure 3.5. The NLS folds onto precisely those residues in the well-structured part of IκBα that undergo backbone dynamics on the µs- ms timescale in the free protein. Such slower motions in one binding partner are thought to be necessary for conformational adjustments that better accommodate the binding partner (Bonvin, et al., 2005). These conformational adjustments may be indicative of “fly-casting” occurring during the binding process (Shoemaker, et al., 2000). Fly-casting refers to the observation that a broader conformational ensemble may “capture” the binding partner more readily thus speeding up the association reaction. Indeed, despite the large and complex interface between NF-κB and IκBα, their association occurs with very rapid kinetics (Bergqvist, et al., 2006).
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References


CHAPTER 4

The nuclear localization sequence peptide of the p65 subunit of NF-κB folds upon binding IκBα
Introduction

The Nuclear factor kappa B (NF-κB) family of transcription factors is key to the control of many cellular signaling events including cellular stress responses, cell growth, survival and apoptosis (Baldwin, 1996, Ghosh, et al., 1998, Gerondakis, et al., 1999). At present, it is known that the NF-κB signaling system is activated by at least 58 viral or bacterial products, 46 stress conditions and chemicals, at least 32 cytokines and receptor ligands, as well as apoptotic mediators and mitogens, resulting in the transcription of more than 150 genes (Pahl, 1999). In resting cells, NF-κB dimers are sequestered in the cytoplasm via their interaction with proteins from the IκB (IκB) family (Karin and Ben-Neriah, 2000). IκBα, the most abundant and well-characterized member of the IκB family, is capable of inhibiting many of the NF-κB family members, including the most abundant NF-κB, p50/p65, and other p65 and cRel-containing homo- and heterodimers (Huxford, et al., 1999). The NF-κB/IκBα complex is highly stable in resting cells, with a half-life of > 8 hours (O'Dea, et al., 2007) However, even a small amount of NF-κB due to leaky inhibition is sufficient to give gene expression; thus, tight regulation of NF-κB nuclear localization is critical for cell homeostasis (O'Dea, et al., 2007, Tergaonkar, et al., 2005).

Regulation of nuclear localization of NF-κB occurs via its NLS sequence, which targets it to the nucleus. IκBα sequesters NF-κB in the cytoplasm by masking the NLS of NF-κB within the binding interface (Baeuerle and Baltimore, 1988, Jacobs and Harrison, 1998). Upon stimulation, IκBα is phosphorylated, ubiquinylated, and degraded, unmasking the NLS of NF-κB allowing translocation to the nucleus and
subsequent transcriptional activation. NLS sequences mediate nuclear import via recognition by the nuclear import receptor (Dang and Lee, 1988, Dingwall and Laskey, 1991). They are generally short 3-20 amino acid sequences rich in lysine and arginine content described by the consensus sequence K-(K/R)-X-(K/R) categorized as either bipartite, containing two clusters of basic amino acids separated by a linker of 10-12 unconserved amino acids as in the nucleoplasmin NLS (KRPAATKKAGQQAKKKKL), or monopartite, containing a single cluster of basic amino acid residues (K/R) as in the simian virus 40 large T antigen (Tag) NLS (PKKKRKV) (Dang and Lee, 1988, Dingwall and Laskey, 1991, Kalderon, et al., 1984, Lanford and Butel, 1984, Leung, et al., 2003). The NLS of the NF-κB family member p65 is a monopartite NLS with the sequence KRKR found at residues 301-304 within the p65 NLS polypeptide (residues 289-321) located the carboxy-terminus of the rel homology domain of p65 (Malek, et al., 1998). In addition to being responsible for targeting NF-κB to the nucleus, the p65-NLS is also partly responsible for the high affinity and specificity of p65 for IκBα (Phelps, et al., 2000, Bergqvist, et al., 2006).

For nuclear localization, the NLS must be exposed for binding to the surface of the nuclear protein-import receptor complex. The crystal structures of the p50/p65 in complex with IκBα and to DNA show electron density for the p65 NLS polypeptide when bound to IκBα but not when bound to DNA, suggesting that the p65 NLS is disordered in the free state and becomes more ordered upon binding to IκBα (Jacobs and Harrison, 1998, Huang, et al., 1997). The inherent flexibility of the NLS polypeptide is thought to afford the advantage of being able to adopt different local structures in order to bind different molecular targets, such as IκBα and Importin a (Dyson and Wright, 2002).
In fact, NLSs bound to Importin a usually adopt extended structures, while the p65 NLS polypeptide adopts a split helical conformation (termed helix3-helix4) when bound to IκBα (Jacobs and Harrison, 1998, Conti and Kuriyan, 2000). Thus, a disordered structure for the free NLS polypeptide would allow it to recognize Importin as well as the various IκB isoforms with the high specificity and affinity that is required for the strict control of NF-κB cellular localization.

The crystal structures of NF-κB (p50/p65) bound to IκBα reveals an extended protein-protein interface formed between IκBα and NF-κB (Jacobs and Harrison, 1998, Huxford, et al., 1998). IκBα contacts NF-κB via its six ankyrin repeat domain and C-terminal PEST region forming multiple contacts with the p65 NLS polypeptide, p50 and p65 dimerization domain and amino terminal domain regions that comprise NF-κB(p50/p65) (Jacobs and Harrison, 1998, Huxford, et al., 1998). The two proteins run antiparallel, with ankyrin repeats 1-3 (AR1-3) contacting the p65 NLS polypeptide, which adopts a helix-turn-helix fold (Jacobs and Harrison, 1998). AR1 is capped by helix four of the NF-κB p65 NLS polypeptide (residues 305-321) via hydrophobic contacts while AR1-AR3 contact helix three of the p65 NLS polypeptide (residues 289-300), via mainly electrostatic contacts(Jacobs and Harrison, 1998). Meanwhile, AR3-6 and the first part of the IκBα PEST sequence contact the NF-κB p50 and p65 dimerization domains and NF-kB p65 amino-terminal domain (Figure 4.1A) (Jacobs and Harrison, 1998, Huxford, et al., 1998).
**Figure 4.1** (A) Ribbon diagram showing the x-ray crystal structure of the NF-κB/IκBα complex. The p50 subunit is colored in green, the p65 RHR and dimerization domain in red, and the p65 NLS polypeptide in magenta. IκBα is colored in grey. (B) Ribbon diagram showing the interactions between the p65 NLS polypeptide and IκBα. IκBα is colored in grey, the p65 NLS polypeptide is colored in magenta, residues involved in hydrophobic interaction are colored in green and residues involved in electrostatic interactions are colored in red and blue. The sequence of the p65 NLS polypeptide highlighting these interactions is shown below a schematic of the secondary structure of the p65 NLS from the x-ray crystal structure. Residues involved in hydrophobic interaction are colored in green and residues involved in electrostatic interactions are colored in red and blue. Residues comprising the NLS signal KRKR are underlined. (C) Prediction of structural disorder in p65 (190-321), which encompasses the dimerization domain. Disorder propensity was predicted via the IUPRED algorithm (Dosztanyi, et al., 2005). Score values above the threshold of 0.5 are considered disordered, while score values below 0.5 are considered ordered. Previous binding thermodynamics and kinetics studies of the NF-κB(p50/p65)/IκBα interaction revealed that the interaction has an extremely low dissociation rate resulting in
very high affinity and the extremely long \textit{in vivo} lifetime of the complex (Bergqvist, et al., 2006). This study also uncovered that the NF-κB(p50/p65)/IκBα interaction depended critically upon the p65 NLS since truncating helix four of the p65-NLS resulted in a more than 1000-fold increase in the dissociation rate and 10,000-fold decrease in the affinity of IκBα for NF-κB (p50/p65). Indeed, helix 4 of NF-κB(p65) was essential for high affinity binding, contributing about half of the binding free energy change observed for the interaction (Bergqvist, et al., 2006). A large negative heat capacity change was observed for the interaction of NF-κB with IκBα that was halved when helix 4 was removed from the NF-κB(p65). This difference was much too large to be accounted for by the small surface area of the NF-κB(p65) helix 4-IκBα interaction, again pointing to a folding-upon binding of the NLS polypeptide to IκBα. However, such a large contribution to the binding free energy from such a small segment of the protein was surprising, which led us to investigate the binding event in more detail.

Previous studies also showed that a peptide corresponding to NF-κB(p65) residues 289-321 by itself bound to IκBα with micromolar affinity. In the present study, we have employed NMR and ITC in order to characterize the structural and dynamics changes undergone by this p65 NLS polypeptide, which is paramount for the specificity and affinity of the NF-κB p65/IκBα interaction, as it transitions from its free state to its IκBα-bound state. Using NMR, we have characterized the NLS polypeptide in both the free and bound states. A truncated form of IκBα which contains the first four ankyrin repeats of IκBα (residues 67-206) has been used as the binding partner, because it is more stable and less susceptible to aggregation than the full ankyrin repeat protein. Our
studies show that the p65-NLS is disordered in the free state but with a weak tendency towards helical structure. The NMR studies show definitively that the NLS folds upon binding to IκBα. While previous studies highlighted helix four of the NLS as being of vital importance to the interaction, we show that the binding hot spot is localized to a single residue, F309, in helix 4 of the p65 NLS.

**Materials and Methods**

*Protein expression and purification.* IκBα(67-206) in pET11a (Novagen) was transformed into the *E.coli* BL21(DE3) strain. Expression of [²H, ¹⁵N] and [²H, ¹³C, ¹⁵N]-labeled IκBα(67-206) was carried out in M9 minimal media in D₂O supplemented with ¹⁵NH₄Cl (2 g/L) and ¹³C-glucose (8g/L). Cells were acclimated by growing them sequentially in 10 mL cultures of M9ZB, M9, M9(50% D₂O) and M9 (90% D₂O). Two one-liter growths were inoculated with the M9(90% D₂O) culture and induced at OD₆₀₀= 0.6 with 0.2 mM IPTG for 24 hours at 18°C. The cells were collected by centrifugation at 5000 rpm for 30 minutes and resuspended in 70 mL/liter of culture of 25 mM Tris (pH7.5), 50 mM NaCl, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 0.3 mM PMSF, and protease inhibitor cocktail(Sigma) and lysed by sonication on ice. The soluble part of the lysate was purified by cation exchange chromatography on a Hi-Load Q-Sepharose 26/10 column (GE Healthcare) using a one hour gradient from 50 to 500 mM NaCl. Final purification of the protein was done on a HiLoad Superdex 75 16/60 gel filtration column (GE Healthcare). Final purification of the protein was done on a HiLoad Superdex 75 16/60 gel filtration column (GE Healthcare). The purified protein was concentrated in
4mL 10K MWCO Amicon concentrators (Millipore) in a fixed angle rotor at 4000 rpm for 15 minute intervals to prevent aggregation.

The C-terminal residues 289–321 and 293-321 of the p65 subunit of NF-κB were expressed in the trp leader vector, which contains a His8tag and thrombin cleavage sequence, and drives small peptides into inclusion bodies (Guttman, et al., 2010). Inclusion bodies were solubilized with 6M guanidine hydrochloride, 50mMTris (pH 7.5) and the solubilized peptide was captured by a Ni-NTA column equilibrated in the same buffer, and a gradient was run to a final concentration of 150mM NaCl, 50 mM Tris (pH 7.5), 2 mM CaCl₂. The peptide was cleaved from the column by incubating with thrombin (0.04 mg) in three column volumes by rocking the column with for 4 h at 25 °C three consecutive times. The final purification step was reverse-phase HPLC on a C18 column (15 µm, 300 X 19 mm I.D.) with a 0–50% acetonitrile gradient, with 0.1% (v/v) TFA. The peptide was lyophilized and dissolved in 25 mM Tris (pH 7.5), 0.5 mM EDTA, 50 mM NaCl and the pH adjusted with 10 M NaOH in 25mM Tris, 0.5 mM EDTA and 50 mM NaCl.

**NMR Experiments and Data Analysis.** The structural and dynamic properties of free and bound p65 NLS were studied by NMS. NMR experiments for sequence-specific resonance assignment p65 NLS (298-321) and (293-321) were carried out at 293 K to obtain sequence-specific assignment of H²N, N, C’, Cα, and Cβ resonances for free and IkBa(67-206)-bound p65 NLS. A combination of HN(CA)CO, HNCO, and HNCACB were collected for the free p65 NLS (293-321) and HNCA, HNCB, HNCO and HN(CO)CACB for free p65 NLS (289-321) and bound p65 NLS (298-321) and (293-321). The details of how each experiment was performed are given in Supplementary
Table 2.

Amide chemical shift perturbation for p65 NLS binding to IκBα was investigated by collecting $^1$H-$^{15}$N HSQC spectra of titration points between a sample of 0.2mM $^{15}$N-p65 NLS(293-321) and 0.2mM $^{15}$N-p65 NLS(293-321)/0.4 mM IκBα(67-206) in order to keep NLS concentration constant. The samples were mixed to give 20 titration points corresponding to IκBα(67-206)/p65 NLS(293-321) molar ratios of 0, 0.0156, 0.0256, 0.0928, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875, 1, 1.125, 1.25, 1.375, 1.5, 1.625, 1.75, 1.875, and 2.

In order to probe the dynamics and structure of p65 NLS in its free and complexed forms, we also collected $^1$H-$^{15}$N heteronuclear NOE saturated and unsaturated spectra measurements for free and bound p65 NLS(293-321) at 293K, recorded in an interleaved manner, as well as fHSQC-CLEANEX-PM experiments. To further characterize the structural and dynamic features of free p65 NLS(289-321), backbone resonance assignment experiments, $^1$H-$^{15}$N heteronuclear measurements and fHSQC-CLEANEX-PM experiments were also acquired at 278K in order to slow down conformational exchange of the free peptide.

Experimental details are reported in Table 4.2 (at the end of the Chapter). Data were processed using NMRpipe (Delaglio, et al., 1995) and analyzed using NMRView (Johnson, 2004) and Curvefit (Mandel, et al., 1995). The secondary structure propensity from the chemical shifts was determined by the approach described (Berjanskii, et al., 2006), with random-coil reference chemical shift values (Wishart, et al., 1995), corrected for primary sequence as described (Schwarzinger, et al., 2001) and deuterium isotope effects for the bound p65 NLS.
Isothermal Titration Calorimetry. ITC experiments were carried out on a Microcal VPITC instrument. IκBα(67-206) purified by size exclusion chromatography S75 column immediately before use. In a typical ITC experiment, 25 injections of 15 µl of 1 mM p65-NLS or 0.05 mM p50/p65 dimerization domain were made into a 0.1mM or 0.005 mM, respectively, of IκBα(67-206) or IκBα(67-287) solution in the cell. ITC experiments were carried out in a buffer of 25 mM Tris (pH 7.5), 0.5 mM EDTA, 50 mM NaCl, 1mM TCEP and 0.5 mM sodium azide at 293K in duplicate. Isotherms were analyzed using the Origin software (Microcal) as described (Wiseman, et al., 1989).

Results

Backbone resonance assignments. The HSQC spectra of free and IκBα-bound p65(289-321) and (293-321) are shown in Figure 2. Backbone resonance assignments of $^{15}$N, $^{13}$C-labeled p65 (289-321) and p65 (293-231) free and in complex with IκBα(67-206) were obtained from a combination of HNCA, HNCB, HNCO and HN(CO)CACB experiments as described in the Methods section. The p65(289-321) NLS peptide was truncated to residue 293, as it yielded a greater number of peaks of more even peak intensity in the complex as shown in Figure 2. This truncation lacks the flexible linker that connects the p65-NLS peptide to the dimerization domain of p65, which could be responsible for the exchange broadening and uneven peak intensity seen in the HSQC spectra of the complex of p65(289-321) with IκBα(67-206)(Figure 2). The backbone assignments for free p65(289-321) and p65(293-321) are shown in Figure 2. As with other unfolded proteins, this peptide is susceptible to aggregation as well as being
extremely sensitive to changes in pH.

Figure 4.2 Backbone resonance assignments of free and bound p65 NLS HSQC spectra. (A) $^1$H-$^{15}$N HSQC spectrum of 0.2 mM $^1$H-$^{15}$N p65(293-321). (b) $^1$H-$^{15}$N HSQC spectrum of $^2$H-$^{15}$N p65(293-321)(0.4 mM)/$^2$H IκBα(67-206)(0.6 mM). Both spectra were collected at 600MHz and 293K.

The HSQC spectra of the free peptides (Figure 2) clearly show that, in the free
form, the NLS peptide is unstructured. In contrast, upon binding, a much greater dispersion of the signals is observed, indicative of a folding upon binding event. In both the free and bound peptide, residues in helix 3 seem to be undergoing exchange broadening. As a result, peaks corresponding to residues 293-296 of the free peptide and 294-295 and 296-297 for the bound peptide could not be assigned.

**Secondary structure of free and bound p65-NLS in solution.** The chemical shift differences between $^{15}N$, $^{13}C\alpha$, $^{13}C\beta$, $^{13}CO$ chemical shifts for free and IκBα(67-206)-bound p65(293-321) and the sequence-corrected random coil chemical shifts are shown in Figure 4.3. The secondary chemical shifts for 293-314 of bound p65(293-321) show the characteristic positive values of $^{13}C\alpha$, $^{13}C\beta$, $^{13}C\alpha$, and $^{13}CO$ (and $^{15}N$ negative values), except for residues 304-305, which are in a turn between helix 3 and helix 4 in the crystal structure of the IκBα/NF-κB complex (Jacobs and Harrison, 1998). Negative values of $^{3}C\alpha$, $^{13}C\beta$, $^{13}C\alpha$, and $^{13}CO$ (and $^{15}N$ positive values) are seen at the C-terminus (residues 315-320) consistent with the lack of secondary structure seen in the crystal structure for this region.

The secondary of $^{15}N$, $^{13}C\alpha$, $^{13}C\beta$, $^{13}C\alpha$, and $^{13}CO$ chemical shifts for free p65(293-321) are indicative of an unfolded peptide. CSI values show a slightly more helical tendency for helix 3 of the free p65-NLS for data collected at 293K (Figure 4.3). Spectra were also collected at 278K for the free p65(289-321) in order to “freeze” the conformational ensemble of the peptide and probe for residual helicity. Under these conditions, the values of the CSI again showed increased helical tendency for helix 3 than helix 4, however, the CSI values are still more characteristic of an unfolded peptide.
Figure 4.3 Representative strip plots for free and bound p65 NLS HSQC spectra. (A) HNCACB and HN(CO)CACB spectra for a 0.3 mM sample of $^{13}$C-$^{15}$N p65(293-321) collected at 501MHz. (B) HNCA and HNCB spectra of $^{2}$H-$^{13}$C-$^{15}$N p65(293-321)(0.4mM)$^{2}$H IκBα(67-206)(0.6mM) collected at 600MHz and 800MHz, respectively. All spectra were collected at 293K.

**Chemical shift perturbation.** The chemical shift differences between the free and IκBα(67-206)-bound forms of p65 NLS(293-321) were calculated according to the
The largest changes seem to correspond to residues located in the turn between helix 3 and helix 4, as well as the ends of helix 3 (in the NLS region of this helix) and helix 4 (residues 313-316) (Figure 4.4). Large changes were also seen in Gly 320 for which multiple peaks are observed, possibly from conformational exchange due to its proximity to proline 321.

Figure 4.4 Assessment of secondary structural preferences of (A) free and (B) IκBα(67-206)-bound p65-NLS. Differences (in ppm) of observed Ca, Ca-Cb, CO, and N chemical shifts with respect to reference values characteristic of the random-coil conformation of small peptides, properly corrected to account for the contributions by sequential neighbors, are shown. Data are from experiments described in Figure 4.3.
Chemical shift differences between the free and IκBα(67-206)-bound p65 NLS were also calculated. The most extreme chemical shift changes are seen in residues seen to form contacts with the p65-NLS in the crystal structure of the complex (Figure 4.5). The corresponding chemical shift changes in IκBα(67-206) upon binding the p65-NLS show that the greatest shifts are seen in residues contacting the p65-NLS polypeptide (Figure 4.6).

**Figure 4.5** Chemical shift differences between the free and IκBα(67-206)-bound p65 NLS (293-321) measured from 1H-15N HSQC spectra obtained at 800 MHz and 293 K.
Figure 4.6 Chemical shift differences between free IκBα(67-206) and the p65 NLS-bound IκBα obtained from HSQC spectra taken at 800 MHz and 298K. The sequence of the IκBα is shown below with the residues involved in the interface underlined and in bold.

**Backbone Dynamics.** $^1$H-$^{15}$N heteronuclear NOE measurements were carried out on the free and IκBα(67-206)-bound p65(293-321) as described in the Methods section. Heteronuclear NOE measurements were also collected for p65-NLS (289-321) at 278K. The heteronuclear NOE values of the bound NLS, shown in closed circles, range from 0.8 to 1, indicative of a well-structured protein, except at the ends where values decrease below 0.5 (Figure 4.7). Heteronuclear NOE values for NLS(289-321) at 278K (closed triangles in Figure 4.7), are higher than those for NLS(293-321) at 293K but lower than
those seen for the complex. Slightly lower heteronuclear NOE values were measured for helix 4 of the complex at 278K in comparison to helix 3. The heteronuclear NOEs for the free NLS(293-321) (open triangles), were below 0.7, characteristic of a disordered protein.

Figure 4.7 [1H-15N] Heteronuclear NOE values for free and IκBα(67-206)-bound p65 NLS. Closed circles indicate data for 2H-15N p65(293-321)(0.4mM)/2H IκBα(67-206)(0.6mM) collected on a 600MHz at 293K. Closed triangles show data collected for a sample of 15N-p65(289-321) at 278K on a 500MHZ instrument. Open triangles indicate data collected for a 0.3mM sample of 15N p65(293-321) at 293K and 600MHz.
**Minor Peaks for the Helix 4 Region of the p65-NLS.** NMR spectra of p65(293-321)/IκBα(67-206) show the presence of minor peaks for residues 309-315 located in helix 4 of the p65-NLS polypeptide, as shown in figure 4.8. Figure 4.9 shows a comparison of strips from 3D HNCA and HNCB spectra of bound p65 NLS(293-321) for major and minor peaks corresponding to residues 309-315.

![Diagram of Helix 3 and Helix 4 in p65-NLS with amino acid sequence]

**Figure 4.8** $^1$H-$^{15}$N HSQC spectrum of $^2$H-$^{15}$N p65(293-321)(0.4mM)/$^2$H IκBα(67-206)(0.6mM) collected at 293K and 600MHz showing assignments for major (black) and minor (red) peaks for residues 309-315 shown.
Figure 4.9 Strip plots from 3D HNCA spectra of $^{[2\text{H},^{13}\text{C},^{15}\text{N}]}$-p65(293-321)(0.4mM)/$^2\text{H}$ IκBα(67-206)(0.6mM) collected at 293K on a 600MHz spectrometer for major and minor peaks of residues 309-315.
Comparison of the secondary structure predicted from chemical shifts for the major (black) and minor (red) peaks shows that both major and minor peaks follow similar secondary structure trends (Figure 4.10). However, lower values are observed for minor peaks compared to major peaks, suggesting decreased helical secondary structure tendencies for the structure that gives rise to the minor peaks.

Figure 4.10 Comparison of secondary structure preferences in major (black) and minor (red) peaks for residues 309-315 of $^2$H-$^{13}$C-$^{15}$N p65(293-321)(0.4mM)$^2$H IκBα(67-206)(0.6mM) calculated as previously described.
In addition to decreased helical secondary structure tendencies, the structure giving rise to the minor peaks also showed increased dynamics as measured by heteronuclear NOE (Figure 4.11). The presence of secondary peaks for this region of the protein suggests an alternate conformation with increased dynamics and decreased secondary structure. Based on the fact that only a single set of chemical shift perturbations were observed in IκBα upon p65-NLS binding, and that these perturbations matched the crystallographically-determined binding site. It is unlikely that the minor peaks correspond to the NLS binding at an alternate binding site and more likely that it corresponds to part of the NLS (mainly helix 4) binding and unbinding from IκBα.

![Helix 3 NLS Helix 4](image)

**Figure 4.11** $^1$H-$^{15}$N heteronuclear NOE values for major (black) and minor (red) peaks of residues 309-315 for $^3$H-$^{15}$N p65(293-321)(0.4mM)$^2$H IκBα(67-206)(0.6mM) collected at 293K on a 600MHz spectrometer.
Hydrogen Exchange Probed by CLEANEX-PM Experiments. In order to further characterize the free state of the p65 NLS-(293-321) we carried out CLEANEX-PM experiments which provide information on exchange processes of amide protons with the solvent, thus providing information on the relative solvent accessibility of different amide protons (Figure 4.12). As expected, the free NLS is characterized by efficient exchange with the solvent, as measured by the increase of cross peaks with increase in the CLEANEX mixing time. In particular, the middle of the peptide (residues 305-307) shows the highest cross peak intensities indicating less protection. According to the crystal structure, these residues correspond to the bend between helices 3 and 4 (Figure 4.1) and their chemical shifts were closer to random coil values (Figure 4.4B). Increased solvent accessibility is also seen for residues 313-316 and around residue 301. In contrast, residues 302-304, 308-312 and 318 show lower CLEANEX ratios indicating a relatively more protected (ie. structured) local environment. Residues 302-304 and 308-311 also correspond to areas of increased helical tendency in the free peptide (Figure 4.4).
Figure 4.12 Ratio of CLEANEX cross peaks at various mixing times from CLEANEX-PM experiment collected on a 0.3mM $^{15}$N p65(293-321) sample on a 501MHz instrument at (A) 278K and (B) 293K.
Mutational Analysis monitored by Isothermal Titration Calorimetry. ITC experiments were performed in order to probe the effect of alanine mutations in the contact residues of helix 4 of the p65-NLS, which contributes some 7.8 kcal/mol to IκBα binding as reported by Bergqvist et al (Bergqvist, et al., 2006). The results of these experiments are summarized in Table 4.1 and Figure 4.13. None of the mutations had a significant effect on the binding affinity of p65(289-321) to IκBα(67-206) except for the F309A mutation, which dramatically decreased binding (Figure 4.14 A, B).

![Figure 4.13](image-url) Summary of binding thermodynamics data for WT and mutant proteins of p65(289-321) and IκBα(67-206) measured by ITC.

In order to quantify the importance of F309 for binding in the context of the dimerization domain of NF-κB, p65(190-321)/p50(248-350), we introduced the F309A mutation in a construct co-expressing p65(190-321)/p50(248-350). Binding isotherms for WT and F309A p65(190-321)/p50(248-350) binding to IκBα(67-287) are shown in Figure 4.14 C, D.
Figure 4.14 ITC binding isotherms for NF-κB p65 constructs in 50 mM NaCl, 25 mM Tris, pH 7.5, 0.5mM EDTA, and 0.5mM sodium azide at 293K. Data were analyzed using a model for a single set of identical binding sites after the heats of dilution of NF-κB into buffer was subtracted. $K_{D,\text{obs}}$ values are shown. (A) WT p65-NLS(293-321) (B) F309A p65-NLS(293-321) (C) WT p65(190-321)/p50(248-350) and (D) F309A p65(190-321)/p50(248-350).
In the context of the full dimerization domain of p65, the F309A mutation did not abolish binding of the dimerization domain with IκBα(67-287) (Figure 4.14 C, D). However, the mutation causes a 30-fold decrease in the binding affinity and enthalpy and a slight decrease in \(-\Delta S\) compared to the WT protein (Table 4.1).

Binding isotherms were also obtained at 50mM and 150 mM NaCl in order to probe the effect of electrostatic contacts, which appear from the crystal structure to mediate the binding of p65-NLS helix 3 to IκBα (Figure 4.1). The effect of ionic strength was fairly small, causing a less than two-fold difference in $K_{D,obs}$ for both p65(293-321) and p65(289-321) (Table 4.1).
Table 4.1 Binding Thermodynamics of IκBα and p65 constructs measured by ITC.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_D$ (nM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$-T\Delta S$ (kcal mol$^{-1}$)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p65 NLS(293-321) (50 nM NaCl)</td>
<td>371 ± 1</td>
<td>-4.171 ± 0.214</td>
<td>-4.454 ± 0.207</td>
<td>-8.222 ± 0.340</td>
</tr>
<tr>
<td>p65 NLS(293-321) (150 nM NaCl)</td>
<td>746 ± 16</td>
<td>-4.266 ± 0.174</td>
<td>-3.956 ± 0.166</td>
<td>-8.221 ± 0.340</td>
</tr>
<tr>
<td>WT p65(289-321) (150 nM NaCl)</td>
<td>1160 ± 13</td>
<td>-3.607 ± 0.363</td>
<td>-4.351 ± 0.435</td>
<td>-7.958 ± 0.798</td>
</tr>
<tr>
<td>WT p65(289-321)</td>
<td>800 ± 64</td>
<td>-3.745 ± 0.027</td>
<td>-4.424 ± 0.117</td>
<td>-8.177 ± 0.144</td>
</tr>
<tr>
<td>Y306A p65(289-321)</td>
<td>2600 ± 40</td>
<td>-2.329 ± 0.012</td>
<td>-5.157 ± 0.088</td>
<td>-7.486 ± 0.100</td>
</tr>
<tr>
<td>F309A p65(289-321)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>I312A p65(289-321)</td>
<td>540 ± 2</td>
<td>-7.347 ± 0.018</td>
<td>-1.090 ± 0.006</td>
<td>-8.437 ± 0.0238</td>
</tr>
<tr>
<td>M313A p65(289-321)</td>
<td>970 ± 49</td>
<td>-2.983 ± 0.018</td>
<td>-5.098 ± 0.041</td>
<td>-8.081 ± 0.059</td>
</tr>
<tr>
<td>F318A p65(289-321)</td>
<td>2700 ± 700</td>
<td>-2.460 ± 0.013</td>
<td>-5.010 ± 0.176</td>
<td>-7.470 ± 0.189</td>
</tr>
<tr>
<td>WT p65(190-321)</td>
<td>0.6</td>
<td>-11.52</td>
<td>-0.80</td>
<td>-12.32</td>
</tr>
<tr>
<td>F309A p65(190-321)</td>
<td>18.5</td>
<td>-8.33</td>
<td>-2.05</td>
<td>-10.38</td>
</tr>
</tbody>
</table>
Discussion

The p65 NLS folds upon binding IκBα. The p65-subunit-containing NF-κB dimers are targeted to the nucleus via a four-residue signal (KRKR, residues 301-304) located in the C-terminal NLS polypeptide of p65 which binds Importin a. IκBα binds and retains NF-κB in the cytoplasm and p65 residues 289-321 were observed for forming two helices with a bend in between them in the crystal structure of the IκBα/NF-κB(p50-p65) complex (Jacobs and Harrison, 1998). Because no electron density was observed for p65 residues 289-321 in the DNA-bound NF-κB(p50/p65) structure, the suggestion was made that the p65-polypeptide might be unfolded in the absence of IκBα (Chen, et al., 1998). Inherent flexibility in this important region of the p65-NLS may be advantageous for binding several targets. The p65 NLS not only binds Importin a, but also various IκB isoforms which retain NF-κB in the cytoplasm. Prediction of disorder propensity using the IUPred algorithm also suggests that the NLS polypeptide of p65 (residues 289-321) is disordered. Folding simulations predicted several folding on binding modes for the NLS polypeptide binding to IκBα and highlighted Ile 298 and R302 as important residues in the binding of helix 3 to IκBα and Phe 309 as important for the binding of helix 4 to IκBα. This latter residue formed both native and non-native contacts to IκBα in the simulations (Latzer, et al., 2007).

In order to investigate the structural and dynamics properties that regulate the biological function of the p65 polypeptide, we performed NMR experiments along with ITC experiments to elucidate the backbone dynamics and thermodynamics of the IκBα/p65-NLS interaction. The results provide the first direct experimental evidence
that the NLS polypeptide folds upon binding IkBα. While the NMR data shows that the free form is clearly in a disordered configuration, the bound form adopts a helical conformation resembling the crystallographically-determined structure. In addition, the peptide goes from being highly dynamic, as expected for a small disordered peptide, to being well-structured in complex with IkBα.

**Phe 309 in Helix 4 of the p65 NLS is critical for the IkBα NF-κB interaction.**

Previous studies showed that a peptide corresponding to NF-κB(p65) residues 289-321 by itself bound to IkBα with micromolar affinity. ITC experiments were used to investigate the contributions to binding of residues within helix 4 of the NLS polypeptide seen to be contacting IkBα in the crystal structure, namely, Tyr306, Phe309, Ile312, M313, and Phe 318. Most of the mutations caused only slight decreases in binding affinity of the p65 NLS for IkBα, however mutation of Phe 309, located in the middle of helix 4, to Ala dramatically decreased the binding affinity. These studies therefore identify the hotspot (Wells, 1996) of this crucial interaction as a single residue, that appears to fit into a hydrophobic pocket on the top face of IkBα formed by Phe 77 and Leu 80 of IkBα (Jacobs and Harrison, 1998). This is in accordance with the prediction that Phe309 would be crucial for binding of helix 4 to IkBα, as it formed several native and non-native contacts with IkBα in folding and binding simulations (Latzer, et al., 2007). In the present study Phe309 emerges as a sort of anchor helping to clamp the helix 4 to IkBα.

It is also interesting that the ΔH of binding of the I312A mutant increases by two-fold while the ΔS decreases thus achieving a similar binding affinity as the wild type
peptide. The decrease in entropy and increase in enthalpy may be due to the substitution of a large side chain, which does not appear to be making any specific contacts in the bound structure, with alanine, which would promote helix formation. It is also interesting that mutation of Phe 318, which is seen reaching towards the IκBα in the crystal structure (Figure 4.1A), had little effect on the binding affinity.

**Significance of the NLS-IκBα interaction.** The folding upon binding of the p65 NLS polypeptide explains the mechanism whereby IκBα-bound NF-κB is retained in the cytoplasm. Although the crystal structure of the NF-κB(p50/p65)•IκBα complex hinted at the fact that the NLS was bound and therefore not accessible to Importin a, the crystallographic B-factors were extremely high, and the affinity of the binding was not known (Jacobs and Harrison, 1998). We now show that the NLS polypeptide undergoes a disorder to order conformational change, effectively masking it from binding by Importin a and inhibiting subsequent translocation to the nucleus. The helical structure induced by IκBα binding is incompatible with the extended structure required for Importin a interaction. Although the binding affinity of the NLS peptide by itself is 1µM, it is effectively held in the bound conformation by the rest of the interaction, for which the overall affinity is 40 pM (Bergqvist, et al., 2006).

We previously showed that the tight binding affinity of the NF-κB(p50/p65)•IκBα complex is encoded in two interactions, one at either end of the large protein-protein interface (Bergqvist, et al., 2006, Bergqvist, et al., 2008). The interaction at the C-terminal part of IκBα involves the weakly-folded ankyrin repeats 5 and 6 of IκBα that fold upon binding the N-terminal part of NF-κB (Truhlar, et al., 2006). We have shown here that at the opposite end of the interface, the C-terminal NLS polypeptide
of p65 NF-κB also folds upon binding IκBα. Thus, the interaction may be said to follow an “I-fold-you, you-fold-me” mechanism. Even though both helix 3 and helix 4 of the NLS polypeptide fold upon binding to IκBα, it is the last helix, helix 4, which has been show to be critical for the interaction (Bergqvist, et al., 2006). Remarkably, we show here that a single residue within helix 4, Phe309, forms a hot spot within this small segment, that appears to button the helix down to cap the top face of the ankyrin repeat domain of IκBα.
Table 4.2 Experimental Details of NMR Experiments on p65-NLS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. (mM)</th>
<th>Field (MHz)</th>
<th>Experiment</th>
<th>Dimension of acquired data</th>
<th>Spectral width (ppm)</th>
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<th>d</th>
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<td>$^{15}$N-$^{13}$C NLS (293-321)</td>
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<td>501</td>
<td>HN(CA)CO</td>
<td>2K (1H) 128 (13C) 32 (15N)</td>
<td>12 (1H) 10 (13C) 22 (15N)</td>
<td>32</td>
<td>0.8</td>
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<tr>
<td></td>
<td></td>
<td>501</td>
<td>HNCO</td>
<td>2K (1H) 128 (13C) 32 (15N)</td>
<td>12 (1H) 10 (13C) 22 (15N)</td>
<td>8</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>501</td>
<td>HNCA B</td>
<td>2K (1H) 128 (13C) 32 (15N)</td>
<td>12 (1H) 64 (13C) 22 (15N)</td>
<td>16</td>
<td>0.9</td>
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<tr>
<td></td>
<td></td>
<td>501</td>
<td>CBCACONH</td>
<td>2K (1H) 106 (13C) 32 (15N)</td>
<td>12 (1H) 64 (13C) 22 (15N)</td>
<td>32</td>
<td>0.9</td>
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<tr>
<td>$^{2}$H-$^{15}$N NLS (293-321)/$^{2}$H-IkBα (67-206)</td>
<td>0.5/0.75</td>
<td>600</td>
<td>HNCO</td>
<td>2K (1H) 90 (13C) 32 (15N)</td>
<td>10 (1H) 66 (13C) 24 (15N)</td>
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<tr>
<td></td>
<td></td>
<td>800</td>
<td>HNCA</td>
<td>2K (1H) 70 (13C) 32 (15N)</td>
<td>10 (1H) 36 (13C) 26 (15N)</td>
<td>112</td>
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<td></td>
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<td>600</td>
<td>HN(CO)CB</td>
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<td>$^{15}$N NLS (289-321) (278K)</td>
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<td>500</td>
<td>hetNOE</td>
<td>2K (1H) 256 (15N)</td>
<td>10 (1H) 30 (15N)</td>
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<td>2</td>
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<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>600</td>
<td>hetNOE</td>
<td>2K (1H) 280 (15N)</td>
<td>9 (1H) 22 (15N)</td>
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<tr>
<td></td>
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<td>HSQC (for CLEANEX-PM)</td>
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<td>CLEANEX-PM (5ms)</td>
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<td>12 (1H) 22 (15N)</td>
<td>16</td>
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<tr>
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<td>0.3</td>
<td>501</td>
<td>CLEANEX-PM (5ms)</td>
<td>2K (1H) 128 (15N)</td>
<td>12 (1H) 22 (15N)</td>
<td>16</td>
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<tr>
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<td></td>
<td>0.3</td>
<td>501</td>
<td>CLEANEX-PM (5ms)</td>
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<td>12 (1H) 22 (15N)</td>
<td>16</td>
</tr>
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<td></td>
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<td>0.3</td>
<td>501</td>
<td>CLEANEX-PM (5ms)</td>
<td>2K (1H) 128 (15N)</td>
<td>12 (1H) 22 (15N)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>501</td>
<td>CLEANEX-PM (5ms)</td>
<td>2K (1H) 128 (15N)</td>
<td>12 (1H) 22 (15N)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>501</td>
<td>CLEANEX-PM (1ms)</td>
<td>2K (1H) 128 (15N)</td>
<td>12 (1H) 22 (15N)</td>
<td>16</td>
</tr>
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<td>$^{2}$H-$^{15}$N NLS (293-321)/$^{2}$H-IkBα (67-206)</td>
<td>0.4/0.6</td>
<td>600</td>
<td>hetNOE</td>
<td>2K (1H) 280 (15N)</td>
<td>10 (1H) 24 (15N)</td>
<td>64</td>
<td>3</td>
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References


interactions with apolipoprotein E. *Biochemistry.* **49**, 1207-1216.


CHAPTER 5

Solution structure and dynamics of the ankyrin repeat domain of wild type and stability mutants of IκBα
Introduction

As has been described in previous chapters, IκBα contacts NF-κB via an ankyrin repeat domain (ARD) that consists of six ankyrin repeats (ARs). IκBα consists of a 317-residue polypeptide containing phosphorylation sites in the N- and C-terminal sections (related to the degradation of the inhibitor), and six ankyrin repeats that function as the binding site for NF-κB. The minimal fragment required for binding and dissociation of DNA-bound NF-κB contains residues K67 to E287 [IκBα(67–287)], encompassing the entire six ARs and the first seven residues of C-terminal PEST sequence (Malek, Huxford and Ghosh, 1998, Phelps, Sengchanthalangsy, Malek and Ghosh, 2000).

The resistance of free IκBα to being crystallized (G.Ghosh, personal communication) suggested that the free protein might be subject to conformational heterogeneity. Biophysical studies showed that free IκBα(67-287) was susceptible to ANS binding suggesting that IκBα(67-287) has molten globule character (Croy, Bergqvist, Huxford, Ghosh and Komives, 2004). AR1–4 fold cooperatively and show protection from amide H/$^2$H exchange that is consistent with compact structure (Ferreiro, et al., 2007, Truhlar, Torpey and Komives, 2006). In contrast, amide exchange experiments show that all of the amides in AR5 and 6 exchange within 2 minutes and do not fold cooperatively (Ferreiro, et al., 2007, Truhlar, Torpey and Komives, 2006). When the solvent accessible surface area (SASA) of IκBα was calculated from the crystal structure of the NF-κB/IκBα complex, it matched the amide exchange with a high correlation coefficient (Figure 5.1A). However, if the structure of the IκBα from the complex was used to calculate the SASA, it matched the amide exchange of AR1-4 but
not AR5-6. These latter two repeats exchanged much more than expected from the SASA indicating that in the unbound state, AR5-6 was not as compactly folded as the structure seen in the complex (Figure 5.1A).

**β-hairpins in ARs 5–6**

*Figure 5.1* (A) The number of deuterons incorporated in each peptide in ARs 1–4 (closed circles) correlates extremely well with the calculated solvent accessible surface area (SASA) of the corresponding region of IκBα in both free and bound. The β-hairpins in ARs 5 and 6 (open circles) in free IκBα exchange to a much greater extent than predicted by their SASA. (Truhlar, Torpey and Komives, 2006)

The reason for the inherent flexibility of AR5 and 6 compared to ARs1-4 may lie in their amino acid sequence. AR consensus sequences based on statistical analyses have been developed in the past years (Kohl, et al., 2003, Mosavi, Minor and Peng, 2002). Based on these, a number of consensus designed AR proteins have been created, which are generally more stable than naturally occurring AR proteins (Kohl, et al., 2003, Mosavi, Minor and Peng, 2002, Mosavi and Peng, 2003, Binz, et al., 2004). Among the consensus sequences, the GXTPLHLA motif (Figure 5.2) is the most prevalent signature. It has been shown that mutations to this consensus stabilize AR domains, while mutations away from it destabilize them (Ferreiro, et al., 2007, Zweifel, Leahy, Hughson and Barrick, 2003, Lowe and Itzhaki, 2007, Tang, Guralnick, Wang, Fersht and Itzhaki, 1999,
Tripp and Barrick, 2007). IκBα deviates from this consensus signature in ARs 1, 2, 4, 5, and 6 (Figure 5.2). While some of these deviations are amino acids that contact NF-κB (F77, Q111, and Q255) (Huxford, Huang, Malek and Ghosh, 1998, Jacobs and Harrison, 1998) many do not contact NF-κB and can be substituted without affecting NF-κB binding (Ferreiro, *et al.*, 2007). For example, mutation of IκBα residues C186 and A220 in ARs 4 and 5, respectively, to consensus residues resulted in a stability increase of ~1.5 kcal/mol (Ferreiro, *et al.*, 2007). Furthermore, mutation of residues Y254 and T257 in AR6 to the consensus appears to “pre-fold” AR5 and 6, increasing the stability of the protein and causing it to have cooperative folding transition, similar to NF-κB-bound IκBα (Truhlar, Mathes, Cervantes, Ghosh and Komives, 2008).

**Figure 5.2** (A) The crystal structure of IκBα (blue) bound to NF-κB (p50, green; p65, red; p65 nuclear localization sequence (NLS), magenta). Residues mutated in previous studies, Y254, T257, C186, and A220, do not contact NF-κB, and they are depicted with ball-and-stick representation and colored cyan. (B) Amino acid sequence of human IκBα showing the positions of the ankyrin repeats and the PEST domain. Positions of secondary-structure elements in the X-ray crystal structure of IκBα in complex with p65(19–321)/p50(248–350) are indicated below the sequence. The positions of the hairpin structures in each repeat are indicated by light blue arrows, and the helices by blue cylinders, with 310 helices shown in white. Residues that correspond to the ankyrin repeat consensus are shown in blue. The sequences of the IκBα ankyrin repeats (ARs) are aligned with the consensus sequence for a stable AR.
These “pre-folding” mutations shed light on the importance of the weakly-structured ARs of IκBα for its function. Free IκBα is degraded by a proteasome-dependent but ubiquitin-independent mechanism. In the cytoplasm, free IκBα has an extremely short lifetime (>10 minutes) (O'Dea, et al., 2007). This process is slower for the pre-folded mutant both in vitro and in cells (Truhlar, Mathes, Cervantes, Ghosh and Komives, 2008). Also, the pre-folded mutant binds NF-κB more weakly as shown by both SPR and ITC in vitro and immunoprecipitation experiments from cells (Truhlar, Mathes, Cervantes, Ghosh and Komives, 2008). The in vivo consequence of the weaker binding is that resting cells containing these mutants show incomplete inhibition of NF-κB activation: they have significant amounts of nuclear NF-κB. Furthermore, the weaker binding combined with the slower degradation rate of the free protein results in reduced levels of nuclear NF-κB upon stimulation. These data clearly demonstrate that the coupled folding and binding of IκBα is critical for its precise control of NF-κB transcriptional activity (Truhlar, Mathes, Cervantes, Ghosh and Komives, 2008).

Due to its conformational heterogeneity, free IκBα(67-287) in solution is extremely prone to aggregation and self association, especially at higher temperature and concentration. This Chapter describes the strategies that have been used to surmount these obstacles in order to conduct solution NMR studies on free IκBα(67-287) in order to probe the heretofore unknown structural and dynamics properties in an amino acid resolution, that are important for the biological function of IκBα. Results on the structural and dynamics properties of free IκBα(67-287), WT and the “pre-folded” Y254LT257A mutant, as probed by solution NMR, are also presented.
Materials and Methods

**Expression and purification of proteins.** General preparation and purification methods for IkBα(67–206) have been described previously in Chapters 2 and 3 and IkBα(67–287) was prepared and purified in the same way. Expression of [\(^2\text{H}, \, ^{13}\text{C}, \, ^{15}\text{N}\)]-labeled IkBα(67–287) or IkBα(67–206) was carried out in M9 minimal medium in D\(_2\)O supplemented with \(^{15}\text{NH}_4\text{Cl} \) (2 g/L), and \(^{13}\text{C}\)-labeled glucose (8 g/L). First, cells were acclimated by growing the cells in at 37°C in 30 mL M9ZB media for 5 hours. These cells were then pelleted and resuspended in 10 mL M9 minimal media and grown for 1 hour at 37°C. 1mL of this culture is then taken and diluted with 1mL of M9 minimal media prepared in D\(_2\)O and grown at 37°C for 2 hours. Finally 18 mL of M9 minimal media prepared in D\(_2\)O are added to the previous culture and grown for 8 hours and then used to inoculate a 1L culture of M9 minimal media prepared in D\(_2\)O and supplemented for labeling as described above. It should be noted that the YLTA mutant does not express very well, so 3L of culture were grown for each prep instead of 1L, as for the WT. Specific \(^{15}\text{N}\)-amino acid growths were grown in M9 minimal medium supplemented with \(^{14}\text{N}\)-labeled amino acids added to the medium in proportion to their abundance in the amino acid sequence to a final total amount of 10g/L culture. In order to prevent scrambling of the label, only 1/10\(^{th}\) of the above amount of the \(^{15}\text{N}\) amino acid was added.

Purification proceeded as described in chapters 2 and 3 except for the HiLoadQ gradient a 1 hour gradient ranging from 225mM to 700mM NaCl was used instead. Final
samples were exchanged into buffer composed of 25 mM 2H-Tris pH 7.5, 50 mM NaCl, 5 mM CHAPS, 2 mM NaN₃ and 2 mM DTT.

**NMR backbone triple-resonance experiments.** Resonance assignments for IkBα free and in complex with NF-κB were made using triply labeled [²H, ¹³C, ¹⁵N] IkBα. HNCA(Grzesiek and Bax, 1992, Yamazaki, Lee, Arrowsmith, Muhandiram and Kay, 1994), and HN(CO)CA(Grzesiek and Bax, 1992, Yamazaki, Lee, Arrowsmith, Muhandiram and Kay, 1994), and HN(CA)CB(Wittekind and Mueller, 1993), and HN(COCA)CB(Yamazaki, Lee, Arrowsmith, Muhandiram and Kay, 1994) and HNCO(Grzesiek and Bax, 1992) spectra, or their TROSY equivalents (Salzmann, Pervushin, Wider, Senn and Wuthrich, 1998), for the longer constructs, were acquired.

For triply labeled [²H, ¹³C, ¹⁵N] IkBα(67–206), NMR spectra were acquired at 20 °C on a Bruker DRX600 spectrometer equipped with a cryoprobe. The sample was exchanged for NMR into the following buffer: 25 mM Tris (pH 7.5)/50 mM NaCl/50 mM arginine/50 mM glutamic acid/5 mM CHAPS/1 mM ethylenediaminetetraacetic acid (EDTA)/1 mM DTT in 90% H₂O and 10% D₂O. The following parameters were used in the 3-D experiments: for HNCA, data size = 2048 (t₃) × 48 (t₂) × 96 (t₁) complex points, number of scans = 8; for HN(CO)CA, data size = 2048 (t₃) × 40 (t₂) × 96 (t₁) complex points, number of scans = 16; for HN(CA)CB, data size = 1024 (t₃) × 32 (t₂) × 90 (t₁) complex points, number of scans = 32; for HN(COCA)CB, data size = 1024 (t₃) × 32 (t₂) × 90 (t₁) complex points, number of scans = 40; for HNCO, data size = 2048 (t₃) × 48 (t₂) × 96 (t₁) complex points, number of scans = 4. The delay time between each scan is 1.5 s.
For (67-287) constructs, NMR spectra were acquired at 20 °C on a Bruker DRX600 spectrometer equipped with a cryoprobe (HNCO, HN(CO)CA) and Avance 800 spectrometer for TROSY spectra (HNCA, HNCB). The following parameters were used in the 3-D experiments: for HNCA, data size = 2048 (t3) × 32 (t2) × 88 (t1) complex points, number of scans = 64; for HN(CO)CA, data size = 2048 (t3) × 32 (t2) × 88 (t1) complex points, number of scans = 24. HNCB=2048 (t3) × 32 (t2) × 90 (t1) complex points, number of scans = 128. The delay time between each scan is 1.2 s.

**NMR relaxation measurements.** Measurements of the $^1$H–$^{15}$N heteronuclear NOE for free IkBα were made at 20 °C with uniformly $[^{15}$N, $^2$H]-labeled IkBα. $^1$H–$^{15}$N NOE measurements, independent saturated and unsaturated spectra were recorded in an interleaved manner for each sample. The parameters used where data size=2048(t3) × 512 (t2), number of scans=128. The delay time between each scan is 3 s. Data were processed using NMRpipe and analyzed using NMRView.

**H/D exchange experiments.** Measurements of H/D exchange for free IkBα were made at 20 °C with uniformly $^{15}$N,$^2$H-labeled IkBα for the (67-206) and (67-287) constructs. H/D exchange was initiated by rapid buffer exchange from H$_2$O into 100% D$_2$O buffer, using an illustra NAP-5 spin column. A series of $^1$H–$^{15}$N HSQC spectra [free IkBα(67–206)] or $^1$H–$^{15}$N TROSY–HSQC spectra [IkBα(67–287)] were acquired every 60 min on Bruker DRX600 or Avance900 spectrometers, respectively, during a total exchange time of 30 h. The first experiments were started within 15 min after buffer exchange. Each exchange experiment was repeated twice.
Results

*Initial NMR characterization of IκBα*(67–287). To obtain site-specific information on the dynamics of free IκBα it is necessary to obtain as complete a set of site-specific resonance assignments as possible. There are several challenges. First, while IκBα is 24.3kDa, which is within the tractable range of sizes by NMR, it is extremely prone to aggregation and self-association. This increases the effective “size” of the protein as perceived by the NMR, as its proclivity toward self-association causes its relaxation properties to resemble those of a much larger protein, resulting in resonance broadening and poor signal in NMR experiments. Secondly, the similarity of structure and consensus sequence in the ankyrin repeats causes a large degree of overlap, requiring confirmation of the assignments with specific labeling or a segmental approach. The HSQC spectrum first acquired for $^{15}\text{N} \text{IκBα}(67-287)$ at 298K and 0.2mM on a 600MHz instrument showed only 140 out of 210 possible peaks as well as non-uniform peak intensities, indicative of differential dynamics amongst the ARs of IκBα. Many resonances were broadened or missing, as shown by the mismatch between residue number and peak number in $^{1}\text{H}–^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) or $^{1}\text{H}–^{15}\text{N}$ transverse relaxation optimized spectroscopy (TROSY)–HSQC spectra (Figure 5.3A).
Figure 5.3 (A) $^1\text{H}-^{15}\text{N}$ HSQC spectrum of 0.2mM $^{15}\text{N}$ IκBα(67-287) at 298K and 600MHz. Buffer conditions were 25mM Tris pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 2mM NaN$_3$ and 1mM DTT. (B) $^1\text{H}-^{15}\text{N}$ TROSY-HSQC spectrum of 0.1mM $^2\text{H}$,$^{13}\text{C}$,$^{15}\text{N}$ IκBα(67-287) at 293K and 800 MHz. (C) Overlay of the spectrum from (B) with that from the NF-κB-bound $^2\text{H}$,$^{13}\text{C}$,$^{15}\text{N}$ IκBα(67-287) collected under the same conditions.

The problem of the larger “effective” molecular weight was ameliorated by adopting TROSY-type pulse schemes collected at high field (800 or 900 MHz) on highly deuterated samples (Salzmann, Pervushin, Wider, Senn and Wuthrich, 1998). TROSY-based experiments effectively reduce the line broadening due to transverse relaxation by selecting for the component for which the different relaxation mechanisms have almost cancelled. Further improvements were obtained by including CHAPS in the sample buffer, use of deuterated Tris, and low protein concentration (0.1mM) and temperature (293K). These approaches resulted in observation of 172/210 expected peaks (Figure 5.3B). While this was the best that could be obtained for WT IκBα(67-287), comparison of this spectrum with that of the bound protein (collected by S. C. Sue under identical conditions) shows binding to NF-κB even further improved the spectrum and all of the
expected peaks are observed for triply-labeled $\text{I} \kappa \text{B}\alpha (67-287)$ bound to NF-$\kappa$B (Figure 5.3C).

**Backbone resonance assignment strategy.** The severe ambiguities in the assignments between the various AR domains were solved with a combination of selective labeling and a segmental approach. Instead of tackling the free WT $\text{I} \kappa \text{B}\alpha (67-287)$ construct directly, an approach utilizing assignments previously made on the truncated $\text{I} \kappa \text{B}\alpha (67-206)$, as well as on the NF-$\kappa$B dimerization domain-bound $\text{I} \kappa \text{B}\alpha (67-287)$ (Sue, Cervantes, Komives and Dyson, 2008), and the stability mutant Y254LT257A were used (Figure 5.4).

![Diagram showing the assignment strategy for free $\text{I} \kappa \text{B}\alpha (67-287)$ based on transfer of assignments from the smaller $\text{I} \kappa \text{B}\alpha (67-206)$, NF-$\kappa$B dimerization domain-bound $\text{I} \kappa \text{B}\alpha (67-287)$ and $\text{I} \kappa \text{B}\alpha (67-287)\text{Y254LT257A}$. The proteins used were $^2\text{H},\text{N},\text{C}$-labeled in order to reduce relaxation and TROSY-type experiments were implemented for the larger constructs and complex(Sue, Cervantes, Komives and Dyson, 2008). Experiments for the free proteins were run at 293K in order to reduce aggregation and self-association and, thus, resonance broadening.](image-url)
Backbone assignments can be transferred to larger proteins and complexes, if the fragments share structural similarity (Sprangers and Kay, 2007, Fiaux, Bertelsen, Horwich and Wuthrich, 2002). This principle applied to the truncated fragment of IκBα since a high degree of overlap was observed for the spectra obtained from IκBα(67-206) and IκBα(67-287) (Figure 5.5). The overlay between these spectra revealed that a portion of IκBα(67-287) (black) is well structured resulting in widely dispersed cross peaks of uniform size. These cross peaks which corresponded closely to the positions of cross peaks seen in the HSQC spectrum of the shorter IκBα(67–206)(blue).

**Figure 5.5** (A) Superposition of the $^1$H–$^{15}$N HSQC spectrum of $[^2]$H, $^{15}$N, $^{13}$C]-IκBα(67–287) (black) and $[^2]$H, $^{15}$N, $^{13}$C]-IκBα(67–206) (blue), showing the overall correspondence between the two spectra. (B) Expanded region of the spectrum of IκBα(67–287) showing broadened peaks in the random coil region of the spectrum (dotted circles).
However, once the overlap between the cross peaks of IκBα(67-287) IκBα(67–206) is accounted for, very few cross peaks remained indicating that still many of the resonances from AR 5 and 6 were missing from the HSQC spectrum of IκBα(67–287). At lower contour levels, a number of weak and broadened resonances appear between 1H chemical shift 7.6 and 9.0 ppm, the central “random coil” region of the spectrum, indicating that a significant part of free IκBα(67–287) is unfolded and/or in intermediate conformational exchange.

In addition to using the assignments from the NF-κB-bound form of IκBα(67–287), the “pre-folded” mutant, YLTA IκBα(67–287), was also used for comparison with the WT protein. Again, a good correlation was observed between the HSQC spectrum of WT (black) and YLTA IκBα(67–287)(red) (Figure 5.6 A). The spectrum of the YLTA mutant strongly suggested that it is “pre-folded”, as a number of new resonances were observed, most notably in the outer regions of the spectra. These resonances most likely correspond to AR5 and 6, as they are located at similar positions as peaks for residues in AR5 and 6 in the spectrum of WT IκBα bound to the NF-κB dimerization domain (Figure 5.6B). Given the good amount of spectral overlap, at least at the HSQC-level, it seemed likely that resonance assignments for AR5 and 6 could be transferred from the spectra of the bound WT protein to the YLTA mutant and finally to the free WT protein.

In addition to good spectral overlap, the IκBα(67-206), YLTA IκBα(67-287) and IκBα(67-287)/p50(248-350)p65(190-321) complex (S.C. Sue) result in better coherence transfer in three-dimensional (3-D) triple-resonance spectra than the WT IκBα(67-287). As expected from overlay of the HSQC spectra, resonance assignments were readily
transferred to the WT protein by analysis of HSQC and triple resonance (HNCO, HNCA, HNCB) spectra. Strip plots illustrating the resonance assignment process are shown in Figure 5.7(A) for AR1-4 residues and Figure 5.7(B) for AR5-6 residues. It is clear that data for the shorter construct has higher S/N, despite the lesser number of scans used for these experiments. Similarly, spectra from both the complex and the mutant protein produced data with higher S/N than spectra from the WT protein. In addition to this data, $^{15}$N selective labeling data (G, V, L, T, and A) were collected, as show in figure 5.8.(Truhlar, Cervantes, Torpey, Kjaergaard and Komives, 2008)
Figure 5.6 (A) Superposition of the $^1$H–$^{15}$N TROSY-HSQC spectra of WT $[^2\text{H}, ^{15}\text{N}, ^{13}\text{C}]$-IκBα(67–287) (black) and YLTA $[^2\text{H}, ^{15}\text{N}, ^{13}\text{C}]$-IκBα(67–287) (blue), showing the overall correspondence between the two spectra. (B) Superposition of the $^1$H–$^{15}$N TROSY-HSQC spectrum of WT $[^2\text{H}, ^{15}\text{N}, ^{13}\text{C}]$-IκBα(67–287) (black) and YLTA $[^2\text{H}, ^{15}\text{N}, ^{13}\text{C}]$-IκBα(67–287) (blue), showing the overall correspondence between the two spectra.
Figure 5.7 Representative strips from the 3D HNCA spectra of free and bound IκBα proteins showing assignment transfer for residues in (A) the variable loop between AR1 and AR2 (B) the variable loop between AR5 and AR6.
Figure 5.8 $^1$H-$^{15}$N HSQC collected at 293K at 800MHz on 0.1 mM selectively labeled samples WT I kBα(67-287).
A large number of the backbone resonances in the YLTA mutant IκBα(67-287) could eventually be assigned (Figure 5.9). Most of the resonances for AR1-5 were unambiguously assigned, including the variable loop connecting AR5 and AR6. The PEST produces signals of extremely strong intensity and was also unambiguously assigned. A few of the resonances preceding the PEST were ambiguously assigned as well. Figure 5.10 shows the TROSY-HSQC spectrum of YLTA with assignments shown.

**Figure 5.9** Sequence of IκBα(67-287) Y254LT257A showing the backbone resonance assignments. Unassigned resonances are shown in black, ambiguous assignments are shown in green and unambiguous assignments are shown in red.
Based on the assignments for IκBα(67-206), the YLTA mutant of IκBα(67-287) and the NF-κB-bound protein, as many assignments as possible were made for WT IκBα(67-287) (Figure 5.11). Resonances for AR5, which could be readily assigned for the YLTA mutant, could not be assigned in the WT protein. Broadening of resonances in the triple resonance data for these peaks, overlap, and also, the large number of missing peaks made assignment of these resonances impossible. However, the variable loop between AR5 and AR6 yielded strong enough data that it could be assigned. As discussed in Chapter 2, peaks for amino acids located in the variable loops of IκBα tend
to be easier to assign because peaks are located in areas of less overlap and tend to give sharper signals, which also facilitated assignment of this variable loop in the free WT protein. Figure 5.12 shows the TROSY-HSQC spectra of WT IkBα(67-287) with assignments labeled. Broadening of the resonances in the center region of the spectrum, can be appreciated by comparing the inset magnifying this region (Figure 5.12) with that from the spectrum of the YLTA mutant protein (Figure 5.10).

Figure 5.11 Sequence of WT IkBα(67-287) showing the backbone resonance assignments. Unassigned resonances are shown in black, ambiguous assignments are shown in green and unambiguous assignments are shown in red.
**Figure 5.12** $^1$H–$^{15}$N TROSY-HSQC spectrum of 0.1mM WT [$^2$H, $^{15}$N, $^{13}$C]-IκBα(67–287) at 600MHZ and 293K with backbone assignments labeled. Inset shows middle region of the spectrum.

**NMR hydrogen–deuterium exchange measurements.** Detection of hydrogen–deuterium (H/D) exchange in NMR experiments provides direct site-specific information on the rate of exchange of amide protons for deuterium, which can be directly correlated with local flexibility of the polypeptide backbone. H/D exchange on the longer constructs of IκBα was performed, as described in Chapter 2, by rapid solvent exchange into buffer prepared in D$_2$O at pH 7.5. Series of TROSY-HSQC spectra for WT IκBα(67–287) and YLTA IκBα(67–287) were acquired at 1 hour intervals after solvent exchange. Experiments were recorded at 293K and 900 MHz. After exchange, only resonances for AR1-4 appeared in the spectra. Residues remaining after 30 hours of exchange are indicated as blue spheres. The results for both proteins are consistent with what was seen
for the 67-206 construct and discussed in Chapter 3. After 3 hours of exchange, resonances could only be seen for the β-hairpins of AR2 and AR3, as well as the outer helix of AR2 (Figure 5.12).

**Figure 5.13** Residues visible immediately after exchange are shown as green spheres on the structure of WT IkBa(67-287) and residues remaining after 30 hours of exchange at 293K are marked as blue spheres for (A) WT IkBa(67-287) and (B) Y254LT257A IkBa(67-287) with the sites of mutation indicated by red spheres.
**NMR relaxation measurements.** Measurement of the $^1$H–$^{15}$N heteronuclear nuclear Overhauser enhancement (NOE) gives information primarily on the picosecond to nanosecond (ps–ns) motions of the polypeptide backbone in the free and bound states. The heteronuclear NOE results are plotted in for WT IκBα(67–287) (black triangles) and the YLTA IκBα(67–287) (red circles) (Figure 5.13). Due to the broadened resonances in the center of the spectrum and the strong resonances of the PEST sequence, several peaks had to be discarded. Heteronuclear NOE values for the first four repeats range between 0.7 and 1 suggesting a well-ordered protein backbone except for the variable lop between AR1 and AR2 where the values drop substantially, particularly the WT protein. AR 5, where data could be measured for the YLTA mutant but not the WT protein due to missing/broadened peaks, shows clearly that this area of the protein has increased dynamics. However, the variable loop between AR5 and AR6 seems to become more ordered as the values of heteronuclear NOE are higher for this area than for AR5 in the YLTA mutant. The PEST regions (residues 281-287) of both proteins also appear to be dynamic, with values at or below 0.5. However, it is interesting to note that the PEST for the mutant is measurably less dynamic than that of the WT protein. Subtraction of the WT heteronuclear NOE values from those of the mutant allowed a direct comparison of the backbone dynamics changes affected by the “prefolding” mutations. For the first repeat, positive values are observed suggesting that the first repeat of the WT protein might be more dynamic than that of the mutant. The AR2–4 region appears to be very similar for both proteins. However, it is clear that the variable loop between AR5 and AR6 and the PEST are appreciably less dynamic in the mutant than in the WT protein.
Figure 5.14 (A) $^1$H-15N heteronuclear NOE values collected on a 600MHX instrument for 0.1mM [$^2$H-15N] samples of WT (black) and Y254LT257A (red) IκBα(67-287). (B) Difference between the heteronuclear NOE values for mutant and WT IκBα(67-287).
Comparison of heteronuclear NOE data for IκBα(67-206), discussed in Chapter 3, with the heteronuclear NOE data for IκBα(67-287) sheds light on the effect of adding two unstable repeats to the end of a well-structured ARD. No significant differences were observed in the first four ARs when the heteronuclear NOE data for the YLTA mutant IκBα (red) was compared to IκBα(67-206) (blue) (Figure 5.14).

Figure 5.15 (A) $^1$H–$^{15}$N heteronuclear NOE values collected on a 600MHX instrument for 0.1mM [$^2$H-$^{15}$N] samples of IκBα(67-206)(blue) and Y254LT257A (red) IκBα(67-287). (B) Difference between the heteronuclear NOE values for mutant and WT IκBα(67-206).

In contrast, comparison of the heteronuclear NOE values for the WT IκBα(67-287) (black) and IκBα(67-206) (blue) revealed differences throughout the protein (Figure
Negative values are mainly observed for residues 67-117, corresponding to AR1 and the variable loop between AR1 and AR2, suggesting that the longer construct is more dynamic than the shorter construct in this region.

**Figure 5.16** (A) \(^1\text{H}^{15}\text{N}\) heteronuclear NOE values collected on a 600MHz instrument for 0.1mM \([^{2}\text{H}^{15}\text{N}]\) samples of IkB\(\alpha\)67-206(blue) and WT (red) IkB\(\alpha\)(67-287). (B) Difference between the heteronuclear NOE values for WT IkB\(\alpha\)(67-287)and IkB\(\alpha\)(67-206).

**Discussion**

Although only incomplete assignments were possible for the WT IkB\(\alpha\)(67-287), enough assignments were made to make comparisons with the pre-folded mutant and
with the well-folded truncated ARD, IκBα(67-206). Both amide exchange and backbone relaxation experiments were pursued to obtain information on the differences in backbone dynamics among these proteins with particular interest in the weakly-folded AR5 and 6. The amide exchange experiments were relatively uninformative since even in the “pre-folded” YLTA mutant IκBα, none of the amides were protected long enough to observe them in the NMR experiments. Mass spectrometry-based amide exchange experiments were able to distinguish that AR5 and 6 were more well-folded in the YLTA mutant than in the WT IκBα, because these experiments allow detection after only seconds of deuteration (Truhlar, Mathes, Cervantes, Ghosh and Komives, 2008).

The heteronuclear NOE experiments turned out to be extremely informative. The fact that no differences were observed between the heteronuclear NOEs measured for the IκBα(67-206) and this same region of the YLTA mutant IκBα(67-287) was reassuring since these two regions were expected to be similarly well-ordered. Comparison of the heteronuclear NOEs measured for IκBα(67-206) and WT IκBα(67-287) on the other hand, gave surprising results. Most notably, long-range increases in backbone dynamics, seen in AR1 and the variable loop connecting AR1 and AR2 are apparently caused by appending two more weakly-folded ARs at the other end of the ARD. Accelerated MD simulations such as those that were discussed in Chapter 2 may be able to shed light on this interesting finding.

Comparison of the WT and “pre-folded” YLTA mutant IκBα(67-287) also gave surprising and unexpected results. Even though the mutations are in AR6, it is very clear that they cause stabilization of the entire AR5. This result helps explain previous results
in which equilibrium folding experiments showed that in the WT protein, Introduction of
the YLTA mutations in AR6 appeared to cause both AR5 and AR6 to be part of the
cooperatively folding ARD because the tryptophan fluorescence of Trp 258 in AR6
followed a cooperative transition in equilibrium unfolding experiments on the YLTA
mutant (Truhlar, Mathes, Cervantes, Ghosh and Komives, 2008). Thus, the Trp
fluorescence experiments confirmed the ordering of AR6 and the heteronuclear NOE
experiments confirm the ordering of AR5, which remained unobservable in the folding
experiments due to the lack of the Trp reporter in AR5.

It is most remarkable, however, that stabilization of AR6 with the YLTA
mutations (at positions 254 and 257) actually stabilize the PEST sequence (residues 277-
287). The entire PEST sequence was assignable in both the WT and the YLTA mutant
IkBα(67-287) and the backbone resonances of all of this region are all significantly more
ordered when the heteronuclear NOE values of the WT are compared to those of the
mutant. This result actually explains what were apparently conflicting earlier
observations. Although Mathes et al had shown that the rate of degradation was
controlled by ordering of the PEST (Mathes, O'Dea, Hoffmann and Ghosh, 2008),
Truhlar et al showed that the YLTA mutant IkBα(67-287) is degraded more slowly than
the WT protein (Truhlar, Mathes, Cervantes, Ghosh and Komives, 2008). These two
results now converge to a common theme, which is that the rate of IkBα degradation is
determined by the foldedness of the PEST sequence.
References


CONCLUSION

Importance of Structural Disorder for Protein function
Evidence of functional structural disorder

The traditional view of the protein structure-function relationship, based on the notion of a definite 3D structure upon which function critically depends, has been challenged in recent years by reports that a large number of proteins contain regions of intrinsic disorder. The importance of protein disorder for function is emphasized by the fact that a large number of unstructured proteins and unstructured segments of proteins are key for signal transduction, cell-cycle regulation, gene expression and chaperone action (Dunker, Brown, Lawson, Iakoucheva and Obradovic, 2002, Ward, Sodhi, McGuffin, Buxton and Jones, 2004, Uversky, 2002, Iakoucheva, Brown, Lawson, Obradovic and Dunker, 2002, Tompa and Csermely, 2004). Thus, the mounting evidence forces a reassessment of the structure-function paradigm to include conformational disorder as a paramount variable of protein function.

The work presented here aimed at investigating the role of structural disorder and conformational heterogeneity for the function of the partially unstructured IκBα ARD and the intrinsically unstructured p65-NLS polypeptide, using NMR spectroscopy as a tool. NMR has proved a powerful tool to give residue-specific information on the conformational heterogeneity of proteins on a wide range of biologically-relevant timescales (ps-ms). Both IκBα and the p65-NLS polypeptide stand to gain functional advantages from having flexible regions. First, both have multiple binding partners. The IκBα ARD binds both p65 homodimers and p50/p65 heterodimers with similar affinities despite having different binding interfaces. The p65-NLS polypeptide must bind both IκB proteins for transcriptional inhibition and Importin a for nuclear transport., that intrinsic flexibility in the free state controls levels of free protein in the cytoplasm.
Flexibility for both p65-NLS polypeptide and the IκBα ARD could provide for the necessarily high specificity for each interaction and a more rapid interaction by, for example, a fly-casting mechanism (ref). Additionally, the levels of free IκBα in the cytoplasm must be tightly regulated for precise control of NF-κB activity. It has been shown and is further corroborated in this thesis.

In Chapters 2 and 3 we discussed the well-structured core comprised of the first four ARs of the IκBα ARD. Although this part of the protein by all means comprises a well-structured protein, the limited µs-ms motions it experiences are significantly found in the very areas that contact NF-κB (p50/p65). Chapter 5 discussed the conformational heterogeneity of the weakly folded AR5 and 6 of the IκBα ARD. Previous studies had shown that the Y254LT257A mutant of IκBα is degraded more slowly than the WT. The work presented here shows that these stabilizing mutations order the PEST sequence despite being ___ amino acids downstream from the site of mutation. It had been shown that the PEST region of IκBα targets free IκBα for degradation by the 20S proteasome. The work presented here shows a correlation between the ns-ps motions of the PEST and its rate of degradation by the 20S proteasome. Finally, Chapter 4 discussed the folding upon binding interaction of the p65-NLS polypeptide and IκBα. NMR studies of this interaction showed that the p65-NLS polypeptide is unstructured, as suggested by previous crystallographic and computational evidence (ref) and folds upon binding IκBα. It has been shown that other NLSs polypeptide bind Importin α in an extended conformation(Fontes, et al., 2003, Fontes, Teh and Kobe, 2000), in contrast to the helix turn helix conformation the p65-NLS polypeptide of unstructured regions in proteins.
which must bind to different targets promptly and accurately, which suggests an advantage for the intrinsic unfoldedness of the p65-NLS. Surprisingly, the p65-NLS polypeptide/ IκBα complex, while extremely important for the binding of the full NF-κB with IκBα, appears to experience disorder at both ends of the interaction. This serves as more evidence to the relatively newly studied phenomenon that structural disorder is important for both protein-protein interactions and in the context of protein complexes(Graham, Ferkey, Mao, Kimelman and Xu, 2001).(Fontes, et al., 2003, Tanaka, Collins, Toyama and Weissman, 2006, Krishnan and Lindquist, 2005, Fontes, Teh, Jans, Brinkworth and Kobe, 2003, Cliff, Harris, Barford, Ladbury and Williams, 2006) Perhaps rigid binding has been evolutionarily compromised in order to provide adaptability, functional flexibility, and reversibility for more precise regulation.

In conclusion, evidence for the importance of structural disorder is increasingly emerging. The work presented in this thesis gives examples of the role of disorder in the function of well-structured proteins, regulatory processes, binding mechanisms and interactomes, which have been mostly disregarded till recently. The cases discussed here join a surge of evidence that is transforming our basic concepts of the protein structure/function paradigm and urges deeper study of the role of the conformational properties of proteins and complexes for fulfilling their biological destinies.
References


