Title
The effect of consensus mutation on the folding and binding kinetics of I(kappa)B(alpha)

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The effect of consensus mutation on the folding and binding kinetics of

I(kappa)B(alpha)

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

in

Chemistry

by

Ingrid L. DeVries

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2011
The dissertation of Ingrid L. DeVries is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2011
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<table>
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<tbody>
<tr>
<td>AR</td>
<td>Ankyrin repeat</td>
</tr>
<tr>
<td>ARD</td>
<td>Ankyrin repeat domain</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>Δ$G_{eq}$</td>
<td>Folding stability</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibiting HIF</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>IκBα</td>
<td>Inhibitor of NFκB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase complex</td>
</tr>
<tr>
<td>$k_a$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>$k_d$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Dissociation equilibrium constant</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSE</td>
<td>Transition state ensemble</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Chapter II, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in *Journal of Molecular Biology*.


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ABSTRACT OF THE DISSERTATION

The effect of consensus mutation on the folding and binding kinetics of

I(kappa)B(alpha)

by

Ingrid L. DeVries

Doctor of Philosophy in Chemistry

University of California, San Diego, 2011

Professor Elizabeth A. Komives, Chair

The NFκB signaling system is important in gene regulation following cellular stress and is linked with numerous cancers and inflammatory diseases. The inhibitor of NFκB, IκBα, holds NFκB in the cytoplasm until cellular signaling initiates the degradation of IκBα. Free NFκB enters the nucleus and up-regulates many genes, including the one for IκBα. Freshly synthesized IκBα actively dissociates NFκB from the DNA, returning to the resting state.

The foldedness of IκBα plays several important roles in NFκB signalling. IκBα is composed of six ankyrin repeats (ARs), two of which, AR5-6, are weakly folded in free IκBα but fully fold upon NFκB binding. The weakly folded nature of AR5-6
allows quick degradation of free IκBα, keeping the cellular concentration of the free inhibitor low and ensuring a fast response when NFκB is activated. The foldedness of AR5-6 is also important in the stripping of NFκB from DNA by IκBα. Here, I examined the foldedness of IκBα by measuring the folding and binding kinetics.

In Chapter 2, I examined the folding kinetics of free IκBα, measuring the kinetics of the well-folded AR1-4 fragment, then comparing the folding kinetics of the full ARD to AR1-4. Using a phi-value analysis, I found that the outer helices of AR3-4 fold first, followed by the outer helices of AR1-2 then finally inner helices. The addition of AR5-6 did not change the folding kinetics, showing that AR5-6 do not contribute to the folding of free IκBα. The bioinformatically-derived ankyrin repeat consensus was also shown to be stabilizing, and will be a useful tool providing potentially infinite stabilization of IκBα.

In Chapter 3, I examined the effect of a hydroxyl modification on the biophysical properties of IκBα. We accurately measured the folding, flexibility, and binding of WT and hydroxylated IκBα and saw that these properties were unaffected by hydroxylation.

In Chapter 4, I studied the coupled folding and binding kinetics of IκBα using a tryptophan probe in AR6. Stabilizing mutations had no influence on the binding kinetics, but introduction of mutations in AR6 that pre-fold AR5-6 resulted in a decreased binding rate, suggesting a fly-casting binding mechanism.
Chapter I

Introduction
The nuclear factor kappa B (NFκB) signaling system is responsible for transcription control of genes involved in cellular events including cell growth and differentiation, the immune response, cell adhesion, and apoptosis (Baeuerle 1998; Ghosh, May et al. 1998; Pahl 1999). Additionally, the NFκB system is important in response to cellular stress and is activated by diverse stimuli including UV light, oxidative stress, inflammatory cytokines, and bacterial and viral products (Baldwin 1996; Ghosh, May et al. 1998; Pahl 1999). Mis-regulation of NFκB has linked with a wide variety of illnesses, including leukemia, lymphoma, and breast cancer, (Yamamoto and Gaynor 2001; Karin, Cao et al. 2002; Shishodia and Aggarwal 2004) as well as chronic inflammatory diseases such as asthma, multiple sclerosis, and rheumatoid arthritis (Tak and Firestein 2001; Yamamoto and Gaynor 2001). The breadth of NFκB signaling presents a great opportunity for new treatments for these illnesses, but also a significant challenge because it is involved in so many pathways. Only by understanding all the nuances of NFκB signaling can specific and effective treatments with minimal side-effects be designed.

The main component in the NFκB signaling system is its namesake, NFκB, an inducible transcription factor. NFκB most commonly refers to a heterodimer of RelA/p65 and p50, through other combinations of p65, p50, cRel, RelB, and p52 also exits (Baldwin 1996; Ghosh, May et al. 1998). NFκB contains a Rel-homology domain, responsible for DNA-binding and dimerization and contains a nuclear localization sequence (NLS) (Ghosh, May et al. 1998). In the resting state, NFκB is
held in the cytoplasm by the inhibitor of NFκB, IκBα, which binds to NFκB and masks the NLS, preventing nuclear import (Jacobs and Harrison 1998). Upon stimulation, IκBα is phosphorylated by the IκB kinase (IKK) complex, then poly-ubiquitinated by the E3IκB ubiquitin ligase complex (Karin and Ben-Neriah 2000), and then rapidly degraded via the 26S proteosome (Ghosh and Karin 2002). The degradation of IκBα releases NFκB, which is then translocated to the nucleus where it activates specific target genes (Ghosh and Karin 2002). Among the target genes of NFκB is the gene encoding IκBα (Ghosh, May et al. 1998). Newly synthesized IκBα enters the nucleus and facilitates the dissociation of NFκB from the DNA (Bergqvist, Alverdi et al. 2009); the complex is then exported from the nucleus, and NFκB is returned to the resting state.

**B. The role of the foldedness of IκBα in NFκB signaling**

IκBα, one of a family of NFκB inhibitor proteins, keeps tight control on the NFκB signaling system (Baeuerle and Baltimore 1988). IκBα contains a N-terminal signal response domain, where the protein is phosphorylated and ubiquitinated, an ankyrin repeat domain (ARD), and a C-terminal PEST domain (Ghosh, May et al. 1998). The ARD is composed of six ankyrin repeats (ARs), each of which contains two antiparallel α-helices connected by a short loop, and then a β-hairpin (Huxford, Huang et al. 1998; Jacobs and Harrison 1998). These repeats stack together to form an elongated domain, stabilized by both inter- and intra-repeat interactions (Huxford, Huang et al. 1998; Jacobs and Harrison 1998). In resting cells, IκBα ARD binds to
NFκB and masks the NLS, preventing nuclear import of NFκB (Karin and Ben-Neriah 2000).

The folding of IκBα is unique among small ARD-containing proteins; the majority of these proteins undergo a single cooperative equilibrium folding (Tang, Guralnick et al. 1999; Zweifel and Barrick 2001; Zeeb, Rosner et al. 2002; Ferreiro, Cho et al. 2005; Lowe and Itzhaki 2007). However, IκBα does not; it undergoes two separate folding transitions, a non-cooperative transition weakly folding AR5-6 and a cooperative transition fully folding AR1-4 (Ferreiro, Cho et al. 2005; Ferreiro, Cervantes et al. 2007). Consequently, only AR1-4 are well folded when IκBα is not bound to NFκB (Croy, Bergqvist et al. 2004; Truhlar, Torpey et al. 2006; Cervantes, Markwick et al. 2009). AR5-6 are weakly folded in the free state and only fully fold on binding to NFκB (Truhlar, Torpey et al. 2006; Sue, Cervantes et al. 2008). This change in foldedness of AR5-6 contributes to many of the ways IκBα achieves such tight control over NFκB.

The change in foldedness of AR5-6 serves as a switch between two degradation pathways. NFκB-bound IκBα undergoes ubiquitin-dependent degradation in response to stimuli (Karin and Ben-Neriah 2000; Ghosh and Karin 2002), while free IκBα undergoes ubiquitin-independent degradation (Mathes, O'Dea et al. 2008). Free IκBα has a cellular half-life of 10 minutes, due to a high degradation rate (O'Dea, Barken et al. 2007). The weakly folded nature of AR5-6 makes IκBα vulnerable to ubiquitin-independent degradation via the 20S proteosome; stabilizing mutations in AR5-6 slow degradation considerably and increase the half-life of IκBα (Truhlar,
Mathes et al. 2008). This rapid degradation maintains low levels of free IκBα, ensuring NFκB does not rebind IκBα once activated.

Conversely, NFκB-bound IκBα has a cellular half-life of over 12 hours when bound to NFκB, suggesting that binding to NFκB stabilizes IκBα (O'Dea, Barken et al. 2007). IκBα and NFκB have a binding affinity of 40 pM (Bergqvist, Croy et al. 2006), due in part to the coupled folding and binding of both AR5-6 and the RelA/p65 NLS (Bergqvist, Croy et al. 2006; Latzer, Papoian et al. 2007; Truhlar, Mathes et al. 2008). High complex stability ensures that the pool of NFκB is ready for activation and, post-activation, that it is rapidly shut down by newly synthesized IκBα.

The foldedness of AR5-6 also contributes to the facilitated dissociation of NFκB from DNA, or stripping. IκBα has been show to increase the rate of dissociation of NFκB from DNA (Bergqvist, Alverdi et al. 2009). Stabilizing mutations throughout the IκBα ARD reduce the stripping ability, but mutations in AR5-6 had the strongest effect (Bergqvist, Alverdi et al. 2009). Recently, it was shown that a stable ternary complex of NFκB, DNA, and IκBα forms (Sue, Alverdi et al.); it is possible that the folding of AR5-6 in the ternary complex results in dissociation of NFκB from DNA.

More and more proteins are being found to have coupled folding and binding. Like IκBα, these proteins are partially or completely unfolded in the absence of their binding partner, but assume a stable fold when bound (Wright and Dyson 2009). One such protein is the antibacterial protein colixin N; the T-domain of colixin N was shown to be highly flexible and largely lacking secondary structure when unbound (James, Kleanthous et al. 1996). The kinetics of binding to its partner TolA, a membrane transporter, were measured by both FRET, following the binding of the two
proteins, and stopped-flow fluorescence, following the folding of colixin N T-domain (Anderluh, Hong et al. 2003). Rates for both events were found to be similar, suggesting a concerted folding and binding mechanism.

Another example is the small signalling protein p21 (Yoon, Venkatachalam et al. 2009). Though p21 is not well folded, it displays an extended conformation in the C-terminal end of the protein, similar of the conformation adopted when bound to the proliferating cell nuclear antigen (PCNA). The N-terminal end of the protein, however, displays helical propensity reminiscent of structure bound to calmodulin. This suggests that bound structures exist in the free protein, which may contribute to high affinity for multiple binding partners.

The tumor suppressor protein, p53, has an unstructured N-terminal domain (Dawson, Muller et al. 2003). This domain was shown to be in an extended, flexible conformation, though it has alternate conformations that interact weakly with other domains of p53 (Huang, Rajagopalan et al. 2009). The unstructured N-terminal domain is responsible for binding with a variety of proteins that regulate p53 activity such as p300/CBP, MDM2, 13-3-3, and S-100 (Huang, Rajagopalan et al. 2009).

The kinase inducible transactivation domain (KID) of the transcription factor CREB also shows coupled folding and binding (Turjanski, Gutkind et al. 2008). While both the unphosphorylated and phosphorylated forms of KID are largely unstructured, there is a slight increase in helicity around Ser133 upon its phosphorylation, which may facilitate binding (Radhakrishnan, Perez-Alvarado et al. 1998). Simulations show that folding and binding are coupled and suggest that KID only becomes structured
after committing to binding, though increasing the residual structure of KID slows binding (Turjanski, Gutkind et al. 2008).

**C. Studying the foldedness of IκBα by stopped-flow tryptophan fluorescence**

Stopped-flow allows real-time observation of kinetics in solution by rapidly mixing two reactants in a mixing chamber where changes in a spectroscopic probe as a function of time can be measured. Coupled with fluorescence, this is a common technique for studying binding events and protein folding.

Protein folding is often studied using intrinsic tryptophan fluorescence, since the change in the environment around the tryptophan changes as the protein folds causes its fluorescence to change (Fersht 1999). Folding kinetics can be studied by stopped-flow tryptophan fluorescence, for which two experiments are performed: refolding and unfolding (Matouschek, Kellis et al. 1990; Jackson and Fersht 1991). For refolding experiments, the protein is equilibrated in denaturant, allowing it to fully unfold. Then, it is rapidly mixed with buffer or a less dilute solution of denaturant, initiating the refolding reaction. For unfolding experiments, the protein is equilibrated in buffer then rapidly mixed with solutions containing high concentrations of denaturant. Kinetic traces are recorded for a range of denaturant concentrations for both experiments and the traces are fit to a sum of exponentials, yielding observed rates for each. These rates are then plotted against the denaturant concentration to create a chevron plot. The chevron plot can reveal numerous details about the folding reactions. The two arms of the chevron can be extrapolated to give folding and
unfolding rates in the absence of denaturant. The slope of each arm gives information about cooperativity. Any curvature in the arms hints at the topology of the transition between folded and unfolded states, including any intermediates (Matouschek, Kellis et al. 1990; Otzen, Kristensen et al. 1999).

Here, stopped-flow fluorescence was used to study the folding kinetics of free IκBα. Because IκBα undergoes two folding transitions, the study was broken down into two parts. First, the cooperatively folded AR1-4 fragment was studied. The folding kinetics of the wild type (WT) AR1-4 were thoroughly examined, then the kinetics of single point mutations based on the AR consensus sequence were compared with the WT. This yielded a detailed folding pathway, showing three distinct steps. The outer helices of AR3 and AR4 fold first, followed by the remainder of AR3 and AR4 in the second step. Lastly, AR1-2 collapse to form the fully folded protein.

With the knowledge of the folding kinetics of AR1-4, the full AR1-6 was analyzed. Mutants characterized in AR1-4 were introduced into the AR1-6 context, and the effect of the folding kinetics measured. This showed that the addition of AR5-6 had no effect on the folding of AR1-4. Additionally, the tryptophan in AR6 showed no contribution to the overall signal change upon unfolding or refolding, confirming that AR5-6 are not well folded in free IκBα.

Stopped-flow fluorescence can also be used to study binding events. Many binding events have been studied by stopped-flow using intrinsic protein fluorescence, including barnase and its inhibitor barstar (Schreiber and Fersht 1993); bacterial toxin colicin E9 Dnase domain and its inhibitor, Im9 (Wallis, Moore et al. 1995); and
cellular receptor, infar2, and interferon α2 (IFNa2) (Piehler and Schreiber 1999). Additionally, stopped-flow fluorescence was used to measure the facilitated dissociation of NFκB from DNA by IκBα using excess unlabeled DNA or IκBα to dissociate NFκB from pyrene-labeled DNA (Bergqvist, Alverdi et al. 2009). More recently, stopped-flow was used to show the formation of a stable ternary IκBα-NFκB-DNA complex using pyrene-labeled DNA (Sue, Alverdi et al.).

Here, we expand on this work by studying the binding of IκBα to NFκB using the intrinsic AR6 tryptophan in IκBα as the fluorophore. This allows direct measurement of the foldedness of AR5-6 during the binding to NFκB. Additionally, the effects on binding kinetics of several stabilizing mutations were measured.

**D. References**


Chapter II

Folding kinetics of the cooperatively folded subdomain of the IκBα ankyrin repeat domain
A. Introduction

IκBα, an inhibitor of the transcription factor, NFκB, contains an ankyrin repeat domain (ARD) with six ankyrin repeats (ARs). IκBα is bound to NFκB in the cytoplasm, until extracellular signals trigger ubiquitin-mediated proteosome-dependant degradation of the IκBα (Baeuerle and Baltimore 1988), allowing NFκB to enter the nucleus and up-regulate transcription of a variety of target genes, among which is the gene for IκBα (Baeuerle and Baltimore 1988). Newly synthesized IκBα then enters the nucleus and interferes with the NFκB-DNA interaction, eventually returning the NFκB to the cytoplasm and the cell to the resting state. It has been shown that IκBα is only fully folded when bound to NFκB; AR5 and AR6 of IκBα are weakly folded in free IκBα (Croy, Bergqvist et al. 2004; Truhlar, Torpey et al. 2006). Upon urea challenge, AR1 through AR4 unfold cooperatively, whereas AR5 and AR6 undergo a non-cooperative transition (Ferreiro, Cervantes et al. 2007).

The folding behavior of AR proteins is interesting because there are no long-range contacts and stabilization is only through local interactions (Ferreiro, Cho et al. 2005). ARs are composed of a β-hairpin, two anti-parallel α-helices (distinguished as “inner” or “helix 1” and “outer” or “helix 2”), and a variable loop. Each repeat makes contacts only within the repeat and with residues of adjacentely stacked repeats. With contacts only a short sequence distance away, it has been proposed that the folding of ARDs is “one-dimensional” (Ferreiro and Wolynes 2008).

The folding of several ARDs has been studied in detail. D34 (Werbeck and Itzhaki 2007) and the Notch (Bradley and Barrick 2002) ankyrin domain, with twelve
and seven ARs each, were analyzed making a single mutation in an analogous position on each repeat. This type of analysis assumes the single position can represent the whole AR unit and, thus, allows direct comparison of the effect of each AR on folding of the ARD. In each Notch AR, the Ala at the start of helix 1 was mutated to Gly, and comparison of the folding kinetics of these mutants showed that Notch folds through an on-pathway intermediate in which the three central ARs were structured (Bradley and Barrick 2006). In each D34 AR, the valine and/or one of the two leucine residues in helix 2 were mutated and comparison of these mutants showed that folding involved a polarized intermediate, in which the C-terminal half of the protein were structured. However, mutation in the C-terminal ARs altered the intermediate, and shifted the folding pathway (Werbeck, Rowling et al. 2008).

The folding kinetics of mutant ARDs were studied in p16$_{\text{INK4A}}$ and myotrophin. In these studies, conservative mutations, generally removing a single methyl group from the side chain of the residue, were examined across the protein sequence to determine how interactions throughout the protein are formed along the folding pathway, allowing a classic phi-value analysis to be performed. The four AR-containing protein, p16$_{\text{INK4A}}$, was found to unfold sequentially, via a polarized transition state in which the two C-terminal ARs were structured but the two N-terminal ARs were not (Tang, Fersht et al. 2003). Similarly, myotrophin folds sequentially, with the C-terminal ARs initiating the folding reaction (Lowe and Itzhaki 2007). In each of these studies, mutations in a particular AR were considered as a group, with the goal of identifying which ARs were folded at the transition state of the folding pathway.
Additionally, the folding p16INK4d (Zeeb, Rosner et al. 2002; Low, Weininger et al. 2007) and tANK (Low, Weininger et al. 2008) were studied with NMR complementing equilibrium and kinetics experiments to obtain residue-specific folding information. Both of these five AR-containing proteins were found to have populated intermediates at equilibrium with the three C-terminal ARs folded.

Several groups have published consensus sequences of the AR motif based on bioinformatic analysis of the hundreds of available AR sequences (Michaely and Bennett 1992; Sedgwick and Smerdon 1999; Mosavi, Minor et al. 2002; Binz, Stumpp et al. 2003; Kohl, Binz et al. 2003; Tripp and Barrick 2007). These sequences differ in the details, but share a core “minimum” consensus with two main regions: one region encompasses the hairpin and the beginning of helix one, the second region covers the end of helix two and part of the variable loop (Figure 2.1a) (Barrick, Ferreiro et al. 2008). In addition, the folding of full-consensus designed ARDs (DARPins) have been studied (Mosavi, Minor et al. 2002; Binz, Stumpp et al. 2003; Kohl, Binz et al. 2003; Devi, Binz et al. 2004; Interlandi, Wetzel et al. 2008; Wetzel, Settanni et al. 2008). Natural ARDs are generally marginally stable, but designed ARD proteins based on the consensus sequence have much higher thermal and chemical stability. Consensus ARDs also fold much faster and unfold much more slowly than naturally occurring ARDs of similar size (Wetzel, Settanni et al. 2008). Naturally occurring ARDs deviate substantially from the consensus: IκBα matches the minimum consensus for just over 50% of residues. IκBα and other large ARDs, such as the Notch ARD, conform more to the consensus in residues 4-12, while smaller ARD-containing proteins, such as p16 and myotrophin, conform better in residues 20-30.
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Figure 2.1. (a) The IκBα_{67-206}W sequence with consensus positions in bold. Tryptophan reporter is highlighted in yellow. Mutated residues are shown in red. Secondary structure is shown below the sequence: the thick arrows represent a β-hairpin and the thick lines represent α-helices. The minimum consensus is represented below the sequence, with highly conserved residues shown and less conserved residues represented by dashes. (b) IκBα_{67-206}W structure, from NF-κB/IκBα_{70-287} structure (PDB: 1NFI) (Jacobs and Harrison 1998). The backbone of the first consensus region encompassing the hairpin and the beginning of helix one is colored green and the backbone of the second consensus region encompassing helix two and the first part of the variable loop is colored blue. A133 is highlighted in yellow. Modifications were performed in PyMol (DeLano 2002). (c) Fraction unfolded from equilibrium urea denaturation of IκBα_{67-206}W (3 µM), monitored by the CD signal at 225, closed circles, or fluorescence, open circles. Lines are from global fits of equilibrium denaturation curves at several temperatures to a two-state model with shared m-values (CD m-value is 2.06 kcal mol^{-1} M^{-1}, fluorescence m-value is 2.12 kcal mol^{-1} M^{-1}).
Guo, et al recently showed that the TPLH motif in the first region of the AR consensus is a major stabilizing factor in central repeats of AR proteins (Guo, Yuan et al.). However, how the consensus residues confer stability on ARDs, and why naturally occurring ARDs deviate so much from the consensus is still not well understood.

Here, we examined the folding kinetics of the first four repeats of IκBα, IκBα_{67-206}, the cooperative folding unit in the IκBα ARD. IκBα_{67-206} displays complex folding behavior, indicative of the presence of on-pathway high-energy intermediates. We also performed mutational analysis, using the AR consensus as a guide to determine the effect of individual consensus positions on folding behavior. Our studies particularly focus on the consensus residues GXTPLHLA, which have not been examined in detail previously. Restoration of a consensus residue resulted in an increase in mutant stability, whereas mutation away from the consensus resulted in decreased stability. Additionally, we found that the majority of mutations affected the unfolding rate, while the only mutations found to affect the folding rate were located in the second helices of AR3 and AR4. By scanning the consensus, we were able to identify parts of a single AR that influence the folding and unfolding rates. We suggest that folding initiates by the formation of the interface of the outer helices of AR3 and AR4, which appears to be folded in the first transition state, while the remainder of the protein folds later. We also show that these conclusions carry over to the full six repeats of IκBα as the presence of ARs 5 and 6 do not appear to affect the folding pathway.
B. Materials and Methods

1. Expression/Purification

IkBα mutations were introduced using QuikChange mutagenesis (Papworth, Bauer et al. 1996). Human IkBα constructs were expressed and purified as described previously (Truhlar, Mathes et al. 2008). The final purification step was on a Superdex-75 gel filtration column (GE Healthcare). The proteins were stored at 4°C and used within 1 week of gel filtration. Protein concentrations were determined by spectrophotometry, using extinction coefficients of 2980 M⁻¹ cm⁻¹ for IkBaα₆₇-₂₀₆, 8480 M⁻¹ cm⁻¹ for IkBaα₆₇-₂₀₆W and mutants; 12950 M⁻¹ cm⁻¹ for IkBaα₆₇-₂₈₇ with a single tryptophan and 18450 M⁻¹ cm⁻¹ for IkBaα₆₇-₂₈₇ with two tryptophans.

2. Urea preparation

Urea (Fisher Scientific, Pittsburg, PA, USA) was dissolved in water, and then treated with AG 501-X8 (D) resin (BioRad Laboratories, Hercules, CA, USA) for 1 hour to remove cyanate contaminants (Street, Courtemanche et al. 2008). Resin was filtered out with a 0.2µm filter and buffer salts were added to the purified urea. Urea concentrations were checked using refractometry (Pace 1986). Urea was used within 2 days of resin treatment to prevent re-accumulation to cyanate.

3. Equilibrium folding experiments

Equilibrium folding experiments were performed with an Aviv 202 spectropolarimeter (Aviv Biomedical, Lakewood, NJ, USA) with a Hamilton Microlab 500 titrator (Hamilton, Reno, NV, USA). A 1 cm fluorescence quartz cuvette containing 2.0 ml of 1–4 µM of the native protein in buffer (10 mM NaHPO₄, 50 mM Na₂HPO₄)
NaCl, 1 mM DTT, 0.5 mM EDTA, pH 7.5) and was titrated with denatured protein (7.3 – 8.4 M urea in buffer), in 30 to 40 injection steps. After each injection, samples were equilibrated with constant stirring at 70-80 rpm for 180s prior to data collection. The CD signal was collected at 225 nm, averaged over 5 to 10 seconds, and the fluorescence signal was collected through a 320 cut-off filter with an excitation wavelength of 280nm, averaged over 2 to 5 seconds. Experiments were performed at 10°C unless otherwise stated.

Equilibrium folding curves were fit to a two state folding model, assuming a linear dependence of the folding free energy on denaturant concentration (Pace 1986). The pre (native) and post (unfolded) transition baselines were treated as linearly dependent on denaturant concentration. The data were globally fit to:

\[
S_{obs} = \left( a_1 + p_1[Urea] \right) + \left( a_2 + p_2[Urea] \right) \exp\left( - (\Delta G - m[Urea]/RT) \right) \\
\left( 1 + \exp\left( - (\Delta G - m[Urea]/RT) \right) \right)
\]

where \( S_{obs} \) is the observed signal, \( p_1 \) and \( p_2 \) are the pre and post transition baselines, \( a_1 \) and \( a_2 \) their corresponding y-intercepts; \( \Delta G \) is the folding free energy in water and \( m \) is the cooperativity parameter (m-value). The data were fit using a non-linear least square fitting algorithm in Kaleidagraph (Synergy Software, Reading, PA, USA) or Profit (QuantumSoft, Uetikon am See, Switzerland).

4. Folding kinetics

Kinetic folding experiments were performed on an Applied Photophysics Pi*-180 stopped flow instrument (Applied Photophysics, Leatherhead, UK). For unfolding experiments, 3 to 10 µM protein in buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, pH 7.5) was rapidly diluted 1:10 with buffered 2.8 to 8.8 M
Urea. For refolding experiments, 3 to 10 µM IkBα67-206 or 1-3 µM IkBα67-287 was unfolded in buffered 4 to 5 M Urea for at least 30 minutes, and then was rapidly diluted 1:10 with buffered 0 to 3 M Urea. Fluorescence was collected perpendicular to the 280 nm emission with a 320 nm cutoff filter over 2 to 200 seconds. At each urea concentration, at 5 to 9 traces averaged and fit to a sum of exponential decays:

\[
\text{Signal} = c + \sum_i A_i \exp(-k_i t)
\]

where \( c \) is the final fluorescence value, \( A_i \) is the amplitudes of the change in fluorescence of each phase, and \( k_i \) the observed rate of folding of each phase.

Observed rates were plotted versus final urea concentration to yield chevron plots. Plots were fit to a four-state model (Bachmann and Kiefhaber 2001; Sanchez and Kiefhaber 2003):

\[
\begin{array}{c}
D \xrightarrow{k_{1,2}} I_1 \xleftarrow{k_{2,1}} I_2 \xrightarrow{k_{3,4}} N \\
\xleftarrow{k_{2,1}} \xrightarrow{k_{3,2}} \xleftarrow{k_{4,3}}
\end{array}
\]

The function and equation are included in the Appendix. Since we have no direct evidence for population of the intermediates, \( k_{2,3} \) and \( k_{3,4} \) were set to 1x10^5 and \( m_{2,1} \) and \( m_{3,4} \) to 0. The data collected at different temperatures were globally fit with shared m-values. All of the data for the different mutants were also globally fit with shared m-values. Fitting of unfolding and refolding traces as well as chevron plots was performed with Profit (QuantumSoft, Uetikon am See, Switzerland).
C. RESULTS

1. Introduction of a tryptophan probe in the cooperatively folding part of IkBα: IkBα_{67-206}A133W

We previously showed that IkBα_{67-206} has similar stability to IkBα_{67-287}, but lacks the steep pre-transition baseline characteristic of the non-cooperative transition attributed to AR5-6 in IkBα_{67-287} (Ferreiro, Cervantes et al. 2007). In order to probe just the cooperative folding transition, a deletion mutant of IkBα was used, containing only the first four ARs: IkBα_{67-206}. A tryptophan residue was engineered into AR2 (A133W) as a fluorescent reporter, as the only natural tryptophan in IkBα is in AR6 (Figure 2.1a, b). The four-AR fragment of the ARD of IkBα recapitulates the entire cooperatively folding unit of the full-length ARD. The A133W mutant, hereafter referred to as IkBα_{67-206}W (Table 2.1), had the same stability as wild type IkBα_{67-206} (Ferreiro, Cervantes et al. 2007), and allowed unfolding to be monitored by both fluorescence and circular dichroism (CD). For IkBα_{67-206}W, both fluorescence and CD equilibrium unfolding curves overlaid well and gave similar stabilities, demonstrating that both the local reporter (W133 in AR2) and the global reporter (CD monitoring helical structure) follow the same two-state cooperative transition (Figure 2.1c).

2. IkBα_{67-206}W folding kinetics

The folding kinetics of IkBα_{67-206}W were measured by stopped-flow fluorescence at 10°C. The decrease in fluorescence induced by unfolding of IkBα_{67-206}W by urea was fit to a single exponential because additional phases did not significantly improve the fit (Figure 2.2a). Refolding experiments showed an increase
Table 2.1. Folding properties of wild type IκBα67-206W at different temperatures

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Equilibrium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Kinetics&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>Temperature</td>
<td>ΔG&lt;sub&gt;eq&lt;/sub&gt;</td>
<td>ΔG&lt;sub&gt;eq&lt;/sub&gt;</td>
</tr>
<tr>
<td>5°C</td>
<td>6.3±0.2</td>
<td>6.5±0.1</td>
</tr>
<tr>
<td>10°C</td>
<td>6.1±0.2</td>
<td>6.2±0.1</td>
</tr>
<tr>
<td>15°C</td>
<td>5.8±0.2</td>
<td>6.0±0.1</td>
</tr>
<tr>
<td>20°C</td>
<td>5.7±0.2</td>
<td>5.9±0.1</td>
</tr>
<tr>
<td>25°C</td>
<td>5.9±0.2</td>
<td>6.3±0.1</td>
</tr>
</tbody>
</table>

Folding stabilities (ΔG<sub>eq</sub>) are reported in kcal mol<sup>-1</sup> and rates in s<sup>-1</sup>.  
<sup>a</sup> Equilibrium denaturation experiments were measured by circular dichroism or total fluorescence. All temperatures were globally fit to a two-state model with baseline drift with shared m-values of 2.06 kcal mol<sup>-1</sup> M<sup>-1</sup> for CD and 2.12 kcal mol<sup>-1</sup> M<sup>-1</sup> for fluorescence. Reported errors are fit errors from the global fit of all temperatures.  
<sup>b</sup> Kinetics experiments were measured by stop-flow fluorescence. All temperatures were globally fit to a four-state model with shared m-values (in kcal mol<sup>-1</sup> M<sup>-1</sup>): m<sub>12</sub> = -0.15; m<sub>21</sub> = 0.96; m<sub>32</sub> = 0.87; m<sub>43</sub> = 0.11; m<sub>23</sub> and m<sub>34</sub> were set to 0 kcal mol<sup>-1</sup> M<sup>-1</sup> and k<sub>23</sub> and k<sub>34</sub> were set to 1 x 10<sup>5</sup> s<sup>-1</sup>. The equilibrium m-value calculated from the m-values for all rate constants is m<sub>eq</sub> = -m<sub>12</sub> + m<sub>21</sub> + m<sub>32</sub> + m<sub>43</sub> = 2.09 kcal mol<sup>-1</sup> M<sup>-1</sup>, in agreement with the equilibrium measurement. To determine experimental error, three independent data sets for wild type IκBα67-206W at 10°C were globally fit with shared m-values. Individual rates were determined for each data set; from these, the standard deviation in each rate was calculated as 14% for k<sub>12</sub>, 22% for k<sub>21</sub>, 22% for k<sub>32</sub>, and 2.6% for k<sub>43</sub>.  
<sup>c</sup> ΔG<sub>eq</sub> was calculated from the individual rates: ΔG<sub>eq</sub> = R T ln (k<sub>43</sub> * k<sub>32</sub> * k<sub>21</sub> / (k<sub>12</sub> * k<sub>23</sub> * k<sub>34</sub>)). Standard deviation in ΔG<sub>eq</sub> was calculated to be 5.7%, based on propagation of the standard deviations of the rates.
Figure 2.2. Refolding and unfolding traces for IκBα_{67-206} W at 10°C, monitored by total fluorescence. Insets show the same data with linear time scale. (a) Folded IκBα_{67-206} W was rapidly mixed to a final urea concentration of 6.5M and the change in fluorescence (of W133) was monitored (●). The unfolding trace was fit with a single exponential function (green line) with residuals shown below the plot. (b) IκBα_{67-206} W, unfolded in 4M urea, was rapidly mixed to a final urea concentration of 0.7M (●). The refolding trace was fit with triple exponential function (green line), with residuals for a double or triple exponential function with residuals shown below for comparison.
in fluorescence induced by rapid mixing of denatured IkBα_{67-206}W with low concentrations of urea. Two to three exponential terms were required to fit the refolding curves (Figure 2.2b), depending on the final denaturant concentration (see below). Importantly, the fluorescence change for both refolding and unfolding experiments has been fully accounted for, indicating that there are no additional kinetic phases taking place within the dead-time of the instrument (Figure 2.3a).

The observed unfolding and refolding rates from single mixing stopped flow experiments were plotted against urea concentration to yield a chevron plot (Figure 2.3c). The plot reveals two distinct regions of unfolding distinguished by a significant change in slope near 5.5M urea. The unfolding phase connects well with one of the refolding phases; this main refolding phase had the largest amplitude of the three refolding phases, accounting for 68-85% of the total amplitude (Figure 2.3b). The main refolding phase showed a strong denaturant dependence. At low urea concentrations, a small downward curvature was observed in the chevron curve.

The slowest refolding phase had the second largest amplitude, accounting for approximately 18% of the total amplitude (Figure 2.3b). This phase was relatively insensitive to denaturant concentration, with a rate of $9.3 \times 10^{-3}$ s$^{-1}$, up to urea concentrations of 2.5M, beyond which the phase was not observed. Interrupted unfolding experiments show that this phase is due to a slow isomerization in the unfolded state (Figure 2.4). The rate constant for this refolding phase becomes faster in the presence of cyclophilin-A, directly showing that folding of these molecules is limited by Xaa-Pro peptide bond isomerization (Figure 2.4). IkBα_{67-206}W has five
Figure 2.3. Folding kinetics of IκBα<sub>67-206</sub>W. (a) Starting (open circles) and ending (closed circles) fluorescence signals for refolding and unfolding traces. (b) Amplitudes of refolding and unfolding phases: unfolding and main refolding phase (closed circles), slow refolding phase (open circles), intermediate refolding phase (open diamonds). (c) Effect of urea concentration on the observed folding or unfolding rate of IκBα<sub>67-206</sub>W at 10°C: unfolding and main refolding phase (closed circles), slow refolding phase (open circles), intermediate refolding phase (open diamonds). Line shows the fit of the data collected at 10°C from the global fit of several temperatures to a four-state model with shared m-values (m<sub>12</sub> = -0.15 kcal mol<sup>-1</sup> M<sup>-1</sup>, m<sub>21</sub> = 0.96 kcal mol<sup>-1</sup> M<sup>-1</sup>, m<sub>32</sub> = 0.87 kcal mol<sup>-1</sup> M<sup>-1</sup>, m<sub>43</sub> = 0.11 kcal mol<sup>-1</sup> M<sup>-1</sup>; m<sub>23</sub> and m<sub>34</sub> were set to 0 kcal mol<sup>-1</sup> M<sup>-1</sup>). (d) Main refolding and unfolding phases for IκBα<sub>67-206</sub>W at 5°C (open squares), 10°C (closed circles, same as in (b) but shown for comparison), 15°C (open diamonds), 20°C (closed triangles), and 25°C (open circles). Lines shown are from the global fit described in (c).
Figure 2.4. Characterization of IκBα67-206 W folding phases  
(a) Interrupted unfolding. IκBα67-206 W (0.25 µM final concentration) was unfolded at 3.9M urea (where IκBα67-206 W unfolds with a rate of 0.36 s\(^{-1}\)) for a set delay time, then jumped to 1.95M urea where refolding was followed by fluorescence. Fluorescence traces were fit with a double exponential function with rates of 0.31 ± 0.02 s\(^{-1}\) and 0.012 ± 0.003 s\(^{-1}\), corresponding to the main phase and the slow phase of IκBα67-206 W refolding. The amplitude for each phase is plotted, with the main phase in red and the slow phase in blue. Both amplitudes were fit with a single exponential function with a rate of 0.009 s\(^{-1}\) for the main phase amplitude and 0.01 s\(^{-1}\) for the slow phase amplitude.  
(b) Effect of Cyclophilin A on refolding rates. Refolding of IκBα67-206 W (0.2 µM) was observed at 0.5M urea in the presence of varying amounts of cyclophilin A. Observed rates of the main refolding phase (red), the slow refolding phase (blue), and the intermediate phase (cyan) are shown.
proline residues, all of which are in the trans conformation in the crystal structures (Huxford, Huang et al. 1998; Jacobs and Harrison 1998). We conclude that the slowest refolding phase corresponds to isomerization-limited folding of unfolded IκBα67-206W molecules with one or more non-native Xaa-Pro peptide bonds (Kiefhaber, Kohler et al. 1992).

A third phase, intermediate in rate, had the smallest amplitude (Figure 2.3b). This phase accounted for approximately 15% of the total amplitude at low urea concentrations, and its amplitude decreased gradually with increasing urea concentration until 2M urea, where it was no longer observed. This phase showed denaturant sensitivity similar to the main phase. It should be noted that the uncertainty in the rates of this phase are high, due to the small separation in rate from the main phase. Cyclophilin A shows little effect on the rate of this phase, suggesting that the reaction is not limited by proline isomerization, however we were unable to obtain any further information on this phase due to its small amplitude. We speculate that this phase may be related to partial folding of IκBα67-206W molecules with non-native Xaa-Pro peptide bonds (Kiefhaber, Kohler et al. 1992) or to isomerization of Xaa-NonPro peptide bonds in the unfolded state (Pappenberger, Aygun et al. 2001).

3. Models to describe the folding kinetics of IκBα

The chevron plot formed by the single IκBα67-206W unfolding phase and the main refolding phase (Figure 2.3c) likely represents the main folding and unfolding event. Since there is a single relaxation, IκBα67-206W folding is a two-state process without populated intermediates. However, the slight curvature at low urea
concentrations and the significant change in slope at high urea concentrations suggests that the transition state region for $\kappa B_{\alpha 67-206W}$ is complex.

Curved chevron plots can be explained by a broad transition state region (Oliveberg 2001). In this case, the curvature is described by a quadratic dependence of the free energies of activation on urea concentrations (Mello, Bradley et al. 2005; Lowe and Itzhaki 2007). This model could not describe the varying degrees of curvature in the chevron plot for $\kappa B_{\alpha 67-206W}$. A three-state folding model with an on-pathway high-energy intermediate (Bachmann and Kiefhaber 2001), which was used to describe the folding of the Notch ARD (Mello, Bradley et al. 2005) and myotrophin mutants (Lowe and Itzhaki 2007), predicts a chevron with a kink only in the unfolding arm (Bachmann and Kiefhaber 2005). This model described the data well at high urea concentrations, but did not account for the curvature in the refolding arm of the chevron. A linear four-state model (Equation 1) with two on-pathway, high-energy intermediates was required to fit the data (Bachmann and Kiefhaber 2001; Sanchez and Kiefhaber 2003).

$$
\begin{align*}
D & \rightleftharpoons_{k_{21}}^1 \leftarrow_{k_{21}} I_1 \rightleftharpoons_{k_{33}}^2 \leftarrow_{k_{33}} I_2 \rightleftharpoons_{k_{34}}^3 \leftarrow_{k_{34}} N
\end{align*}
$$

Equation 1

In this model, the two intermediates are of higher free energy than both the unfolded and the folded state, and do not populate to a detectable amount. Fitting such a model to the data provides information about the relative free energies of the transition state ensembles (TSEs) flanking the intermediates but not on the free energy of the intermediates themselves. To account for this, for data fitting, $k_{23}$ and $k_{34}$ were fixed to $1 \times 10^5$ s$^{-1}$ and $m_{23}$ and $m_{34}$ were fixed at 0 kcal mol$^{-1}$ M$^{-1}$. The linear four-
The state model describes the main folding reaction of IκBα67-206W at 10°C well (Figure 2.3c, Table 2.1).

4. Effect of temperature

The folding kinetics of IκBα67-206W were examined at several temperatures. The observed refolding and unfolding rates increased across urea concentrations with temperatures from 5°C to 25°C while retaining similar slopes (Figure 2.3d). As the temperature increased, the curvature observed in the refolding rates increased. The chevron plots at each temperature were globally fit using the four-state model with shared m-values (Figure 2.3d). At all temperatures, the folding DGs calculated from the folding and unfolding rates agreed with stabilities obtained from equilibrium experiments which were separately globally fit to a two-state model (Table 2.1). Thus, the four-state model can describe the folding kinetics of IκBα67-206W at different conditions. This result shows that the apparent changes of slope in the chevron plot are due to change in the microscopic rate constants for an otherwise robust mechanism of IκBα67-206W folding.

5. Consensus mutations

To probe the effect of sequence on the folding behavior of IκBα67-206W, single mutations were made to consensus positions in the protein (Figure 2.1a). Where the IκBα67-206W sequence diverged from the consensus, the consensus was restored (e.g., Q111G); where the IκBα67-206W sequence agreed with the consensus, conservative mutations were made (e.g., T113S). All mutants were expressed and purified, except L130V, G155A, and L202V, which could not be purified in monomeric form; the
stabilities and folding kinetics were then measured and compared to $\text{IκB}_\alpha_{67-206}$ W. In total, 19 mutations were introduced, and of these, 16 yielded soluble protein that could be analyzed for folding kinetics and stability.

Initially, the stability of each mutant was determined by equilibrium urea denaturation, measured by CD or fluorescence (Table 2.2). As with $\text{IκB}_\alpha_{67-206}$ W, there was good agreement between the two probes. This shows that the two-state mechanism for $\text{IκB}_\alpha_{67-206}$ W equilibrium denaturation is robust to changes in sequence. Some mutants had considerable change in stability compared to $\text{IκB}_\alpha_{67-206}$ W. V93L, Q111G, N122G, A127V, T164L, C186P, and V203L all had significantly higher stabilities than $\text{IκB}_\alpha_{67-206}$ W, while T113S, L117V, L131V, T146S, L163V, T185S, and G194A had lower stabilities.

To investigate the mutants further, we performed stop-flow refolding and unfolding kinetics experiments to obtain chevron plots. It was possible to categorize the chevron plots into three different types (Figure 2.5). Two mutants, both mutations towards the consensus, (S76T/F77P double mutant and V160A) did not differ significantly from wild type (Figure 2.5a, j). The main phase of each chevron plot was globally fit with the four-state model using shared m-values (Figure 2.5, Table 2.2). In the following sections, we compare the results with $\text{IκB}_\alpha_{67-206}$ W (hereafter referred to as WT) according to how each mutation affected the folding kinetics.

6. Only AR3 and AR4 mutations affected refolding kinetics

Of 16 mutants analyzed, only five showed more than a two-fold change in the $k_{12}$: L163V, T164L, T185S, G194A, and V203L (Table 2.2). L163V, a mutation away from the consensus in helix 2 of AR3, decreased $k_{12}$ from 2.4 s$^{-1}$ for the WT protein to
**Table 2.2.** Folding properties of IκBα mutants

**A. Folding properties of IκBα<sub>67-206</sub>W mutants**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>ΔG&lt;sub&gt;eq&lt;/sub&gt;</th>
<th>ΔG&lt;sub&gt;eq&lt;/sub&gt;</th>
<th>k&lt;sub&gt;12&lt;/sub&gt;</th>
<th>k&lt;sub&gt;21&lt;/sub&gt;</th>
<th>k&lt;sub&gt;32&lt;/sub&gt;</th>
<th>k&lt;sub&gt;43&lt;/sub&gt;</th>
<th>ΔG&lt;sub&gt;eq&lt;/sub&gt;</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>IκBα&lt;sub&gt;67-206&lt;/sub&gt;W</td>
<td>6.5±0.2</td>
<td>6.9±0.1</td>
<td>2.4</td>
<td>2.4 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>57</td>
<td>0.71</td>
<td>5.7</td>
<td>dashed line</td>
</tr>
<tr>
<td>S76T/F77P</td>
<td>6.9±0.2</td>
<td>7.5±0.1</td>
<td>3.0</td>
<td>2.1 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>51</td>
<td>0.55</td>
<td>6.1</td>
<td>2.5a</td>
</tr>
<tr>
<td>V93L</td>
<td>8.6±0.2</td>
<td>9.1±0.1</td>
<td>2.0</td>
<td>0.91 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>13</td>
<td>0.25</td>
<td>7.5</td>
<td>2.5b</td>
</tr>
<tr>
<td>Q111G</td>
<td>8.4±0.2</td>
<td>9.2±0.1</td>
<td>3.7</td>
<td>2.0 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4.7</td>
<td>0.30</td>
<td>7.9</td>
<td>2.5c</td>
</tr>
<tr>
<td>T113S</td>
<td>5.6±0.2</td>
<td>6.1±0.1</td>
<td>2.1</td>
<td>1.3 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>68</td>
<td>1.3</td>
<td>5.5</td>
<td>2.5d</td>
</tr>
<tr>
<td>L117V</td>
<td>5.5±0.2</td>
<td>5.8±0.1</td>
<td>2.2</td>
<td>1.4 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>89</td>
<td>1.7</td>
<td>5.2</td>
<td>2.5e</td>
</tr>
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Table 2.2. Folding properties of IκBa mutants, continued

B. Folding properties of IκBa_{67-287}W mutants

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<th>Kineticsb</th>
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<td>V203L</td>
<td>7.4±0.3</td>
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Folding stabilities (ΔG_{eq}) are reported in kcal mol⁻¹ and rates in s⁻¹.  
* Equilibrium denaturation experiments measured by circular dichroism or total fluorescence. All IκBa_{67-206}W mutants were globally fit to a two-state model with baseline drift with shared m-values of 2.19 kcal mol⁻¹ M⁻¹ for CD and 2.35 kcal mol⁻¹ M⁻¹ for fluorescence. IκBa_{67-287}W mutants were also globally fit with shared m-values of 2.13 kcal mol⁻¹ M⁻¹ for CD and 2.20 kcal mol⁻¹ M⁻¹ for fluorescence. Reported errors are fit errors from the global fit of all mutants.  
b Kinetics experiments were measured by stop-flow fluorescence. All IκBa_{67-206}W mutants were globally fit to a four-state model with shared m-values (in kcal mol⁻¹ M⁻¹): m_{12} = -0.11; m_{21} = 0.98; m_{32} = 0.89; m_{43} = 0.11; IκBa_{67-287}W mutants were similarly globally fit to a four-state model with shared m-values (in kcal mol⁻¹ M⁻¹): m_{12} = -0.15; m_{21} = 0.96; m_{32} = 0.87; m_{43} = 0.11. For both, m_{23} and m_{34} were set to 0 kcal mol⁻¹ M⁻¹ and k_{23} and k_{34} were set to 1 x 10⁵ s⁻¹. The equilibrium m-value calculated from the m-values for all rate constants is m_{eq} = m_{12} + m_{21} + m_{32} + m_{43} = 2.10 kcal mol⁻¹ M⁻¹ for IκBa_{67-206}W mutants and 2.09 kcal mol⁻¹ M⁻¹ for IκBa_{67-287}W mutants, both in agreement with the equilibrium measurement. To determine experimental error, three independent data sets for wild type IκBa_{67-206}W at 10°C were globally fit with shared m-values. Individual rates were determined for each data set; from these, the standard deviation in each rate was calculated as 14% for k_{12}, 22% for k_{21}, 22% for k_{32}, and 2.6% for k_{43}.  
c ΔG_{eq} was calculated from the individual rates: ΔG_{eq} = R T ln (k_{43} * k_{32} * k_{21} / (k_{12} * k_{23} * k_{34})). Standard deviation in ΔG_{eq} was calculated to be 5.7%, based on propagation of the standard deviations of the rates.
Figure 2.5. Chevron plots for IκBα<sub>67-206</sub>W mutants showing observed unfolding and major refolding rates at 10°C. Lines shown are from the global fit of WT IκBα<sub>67-206</sub>W and all mutants to four-state model with shared m-values ($m_{12} = -0.11 \text{ kcal mol}^{-1} \text{ M}^{-1}$, $m_{21} = 0.98 \text{ kcal mol}^{-1} \text{ M}^{-1}$, $m_{32} = 0.89 \text{ kcal mol}^{-1} \text{ M}^{-1}$, $m_{43} = 0.11 \text{ kcal mol}^{-1} \text{ M}^{-1}$; $m_{23}$ and $m_{34}$ were set to 0 kcal mol$^{-1}$ M$^{-1}$). The WT IκBα<sub>67-206</sub>W fit is shown in all plots for comparison (black dashed line). Mutants shown are: (a) S76T/F77P (dark grey); (b) V93L (pink); (c) Q111G (cyan); (d) T113S (dark purple); (e) L117V (tan); (f) N122G (magenta); (g) A127V (brown); (h) L131V (light green); (i) T146S (light purple); (j) V160A (brown); (k) L163V (blue); (l) T164L (dark red); (m) T185S (green); (n) C186P (orange); (o) G194A (red); (p) V203L (light grey).
0.65 s\(^{-1}\), and was destabilized by 0.78 kcal mol\(^{-1}\) (Figure 2.5k). Restoring the consensus, T164L also showed a strong effect by increasing \(k_{12}\) to 9.7 s\(^{-1}\). This mutation stabilized the protein by 1.4 kcal mol\(^{-1}\) (Figure 2.5l).

In AR4, the T185S mutation away from the consensus increased \(k_{12}\) to 4.0 s\(^{-1}\) but also increased the unfolding rates and did not show an overall affect on \(\Delta G\). The G194A mutation, between helices 1 and 2, slowed refolding, decreasing \(k_{12}\) to 1.0 s\(^{-1}\), and destabilized the protein by 0.6 kcal mol\(^{-1}\) (Figure 2.5o). Finally, the V203L mutation, restoring the consensus in helix 2 of AR4, increased \(k_{12}\) to 4.0 s\(^{-1}\) without affecting the overall unfolding rate, resulting in a stabilization of 1.5 kcal mol\(^{-1}\) (Figure 2.5p).

### 7. Most mutations had altered unfolding kinetics

The majority of mutations mainly caused changes in the unfolding rates (Figure 2.5, Table 2.2). Three mutations, V93L (Figure 2.5b), Q111G (Figure 2.5c), and C186P (Figure 2.5n), all towards the consensus, unfolded more slowly than WT; fits of the chevron plots also showed slower unfolding rates (\(k_{21}\), \(k_{32}\), and \(k_{43}\)) than WT, resulting in stabilization of the proteins by 1.8, 2.2 and 0.6 kcal mol\(^{-1}\) respectively.

Two additional mutations, N122G (Figure 2.5f), and A127V (Figure 2.5g), slowed \(k_{21}\) and \(k_{32}\) but not \(k_{43}\). Both these mutants were also stabilized by 0.9 kcal mol\(^{-1}\).

Faster unfolding rates were observed for five mutants: T113S (Figure 2.5d), L117V (Figure 2.5e), L131V (Figure 2.5h), T146S (Figure 2.5i), and T185S (Figure 2.5m), all mutations away from the consensus. Of these mutations, all but T185S increased \(k_{43}\) and \(k_{32}\) and they destabilized the protein by 0.2, 0.5, 0.4, and 0.8,
respectively. The T185S mutation increased $k_{d3}$ but decreased $k_{32}$ and also increased the refolding rate resulting in no overall change in stability.

8. Folding of the 6AR protein and mutants

We showed previously that the naturally occurring W258 in AR6 does not follow the cooperative unfolding transition and we speculated that AR5-AR6 do not contribute to the cooperative folding of IkBa$_{67-287}$ (the full 6ARs) (Ferreiro, Cervantes et al. 2007). To directly test this speculation, we introduced the A133W mutation into IkBa$_{67-287}$ and silenced the W258: IkBa$_{67-287}$ A133W/W258F. This construct allowed direct comparison of the behavior of A133W fluorescence within the four- and six-AR constructs. Since IkBa$_{67-287}$ is more aggregation prone than its four-AR counterpart, we choose to perform these experiments at a lowered temperature of 5°C. Experiments were performed at several concentrations from 1-3 µM IkBa$_{67-287}$ and no concentration dependence of the folding kinetics was observed. Unfolding experiments with IkBa$_{67-287}$ A133W, which still contains W258, and the single tryptophan-containing IkBa$_{67-287}$ A133W/W258F revealed one or two folding phases while refolding experiments showed two to four phases, similar to the four-AR protein. Despite the difference in rates, the chevron plots for both six-AR-containing proteins were remarkably similar to the four-AR IkBa$_{67-206}$ A133W (Figure 2.6, Table 2). We introduced three mutations studied in the 4AR protein to determine if the mutations would have the same effect in the 6AR protein (Table 2.2). We chose mutants with a large stabilizing effect on the 4AR protein: Q111G, which decreased the unfolding rate (Figure 2.7a, b), T164L, which increased the refolding rate (Figure 2.7c, d) and V203L, which also increased the refolding rate (Figure 2.7e, f). These mutations in the 6AR context
Figure 2.6. Chevron plots for IkBa_{67-287} A133W/W258F (red circles) and A133W (black triangles) showing main unfolding and refolding rates at 5°C. Solid lines show global fits of all six-AR mutants to a four-state model with shared m-values ($m_{12} = -0.15$ kcal mol$^{-1}$ M$^{-1}$, $m_{21} = 0.96$ kcal mol$^{-1}$ M$^{-1}$, $m_{32} = 0.87$ kcal mol$^{-1}$ M$^{-1}$, $m_{43} = 0.11$ kcal mol$^{-1}$ M$^{-1}$; $m_{23}$ and $m_{34}$ were set to 0 kcal mol$^{-1}$ M$^{-1}$). For comparison, the fit of four-AR IkBa_{67-206} W (main phase at 5°C) to four-state model is shown as a dashed line.
Figure 2.7. Chevron plots for IκBα A133W mutants showing observed unfolding and refolding rates at 5°C (six-AR) and 10°C (four-AR). (a) Four-AR IκBα_{67-206} A133W Q111G, (b) six-AR IκBα_{67-287} A133W Q111G, (c) four-AR IκBα_{67-206} A133W T164L, (d) six-AR IκBα_{67-287} A133W T164L, (e) four-AR IκBα_{67-206} A133W V203L, (f) six-AR IκBα_{67-287} A133W V203L. Lines show global fits of all four-AR or six-AR mutants to a four-state model with shared m-values (as described in Figure 2.5 and Figure 2.5) of WT (dashed line) and mutants (solid lines). The color scheme for the individual mutations is the same as was used in Figure 2.5.
showed similar affects as had been seen in the 4AR context again suggesting that the presence of AR5-6 does not strongly affect the main folding route of the full-length ARD. The effects of the Q111G and T164L mutations were quantitatively similar in the four-AR and six-AR context, but the effect of the V203L mutation was less in the six-AR context. This result is most likely due to the proximity of the V203L mutation to the additional repeats.

D. Discussion

IκBα67-206W shows a simple, two-state mechanism for equilibrium denaturation that yet has complex folding kinetics; the chevron plot was non-linear at both low and high urea concentrations, inconsistent with a simple two-state folding model. Simple folding models that may account for non-linearity including a two-state model with broad transition state ensemble and a three-state model with a metastable intermediate proved insufficient. On the other hand, a four-state model with two high-energy intermediates fully accounted for the chevron plots of the wild type IκBα67-206W at all tested temperatures and the chevron plots for all mutants. In addition, folding DG values obtained from globally fitting all of the mutant equilibrium unfolding curves to a two-state model and those obtained from globally fitting all of the kinetic data agreed well.

1. Consensus stabilization

The folding kinetics of 16 mutants of IκBα67-206W were analyzed and compared; of these, eight conservative mutations were made at positions conforming to the consensus (T113S, L117V, L131V, T146S, V160A, L163V, T185S, and
G194A), and all except V160A and T185S destabilized IκBα\textsubscript{67-206}W. Eight mutations (S75T/F77P, V93L, Q111G, N122G, A127V, T164L, C186P, and V203L) restored the consensus at positions not in agreement with the consensus; all of these stabilized IκBα\textsubscript{67-206}W. Previous studies on IκBα\textsubscript{67-287}, a longer construct containing all six ARs of IκBα, also reported that Q111G and C186P stabilized (Ferreiro, Cervantes et al. 2007). These results suggest a strong correlation between the ankyrin consensus and the folding stability of IκBα\textsubscript{67-206}W. For every mutation that changed the folding stability of IκBα\textsubscript{67-206}W, the construct with the consensus residue was always more stable. This result is consistent with the observation that full consensus AR proteins have very high stabilities (Mosavi, Minor et al. 2002; Devi, Binz et al. 2004; Wetzel, Settanni et al. 2008). Although it was known that full consensus proteins are more stable, a comprehensive analysis of every consensus position has not been studied. The stability of the full consensus protein could have been because one or two of the consensus amino acids were responsible for stability while the others had some other function. Here, we specifically probed the consensus positions and our results show that each and every consensus position, one at a time, imparts stability. Furthermore, the results show that the consensus residues have differential effects on folding kinetics depending on their location within a single repeat.

2. Effect of mutations on folding

The effect of mutations on the folding and unfolding rates of IκBα\textsubscript{67-206}W can be used to develop a picture of the folding pathway. Phi-value analysis is a useful tool for analyzing the change in folding rate upon mutation as compared with the change in stability (Fersht, Matouschek et al. 1992):
Mutants that change the stability of the protein but do not affect the folding rate have a $\Phi_F$ value of 0, suggesting that the contacts to this residue are not formed yet in the transition state. Conversely, mutants that change the stability of the protein solely because of a change in folding rate have $\Phi_F$ value of 1. Applying phi-value analysis to a set of mutations throughout a protein can give structural information about the transition state for folding, and can show which regions or structural features fold first.

Phi-value analysis on a more complex folding pathway such as the one we observed for IkB$\alpha_{67-206}$W can also be performed on the individual transition state ensembles (TSEs) in the folding reaction. The folding rate to each TSE was calculated and used to determine the DDG for each TSE. To determine the average degree of folding in each TSE, we performed a Leffler analysis on the DDGs of each TSE (Figure 2.8, Table 2.3) (Fersht, Matouschek et al. 1992; Itzhaki, Otzen et al. 1995). We found it useful to plot the DDG values for residues in AR 3 and 4 separately from those in AR 1 and 2 and to fit each separately. The results revealed that the slope of the Leffler plot for AR 1 and 2 was near zero for TSEs 1 and 2, corresponding to an average phi-value close to zero. On the other hand, the slope for AR 3 and 4 was 0.45 for TSE1 and 0.79 for TSE2, indicating a progressive folding of this region along the reaction coordinate. This differentiation between N- and C-terminal repeats is made
Figure 2.8. Leffler plots of $\Delta \Delta G_{TSE}$ compared to $\Delta \Delta G_{equilibrium}$ for $\text{IκB} \alpha_{67-206} W$ mutants for (a) TSE 1, (b) TSE 2, and (c) TSE 3. AR 1 and 2 mutants (open squares) are shown with linear fits of these mutants (long-dashed lines), with slopes of 0.03, 0.07, and 0.70, corresponding to the average phi-values for AR 1 and 2 mutants in the three TSEs. AR 3 and 4 mutants (closed diamonds) are shown with linear fits of these mutants (short-dashed lines), with slopes of 0.45, 0.79, and 0.95, corresponding to average phi-values for AR 3 and 4 mutants in the three TSEs. For reference, solid lines of slope 0 and 1 are shown, corresponding to average phi-values of 0 and 1. (c) Average phi-value versus $\alpha$, the location of each TSE along the reaction coordinate, for AR 1 and 2 mutants (closed circles) and AR 3 and 4 mutants (open circles). $\alpha$ was calculated globally as 0.05, 0.52, 0.95 for $\text{IκB} \alpha_{67-206} W$ and 0.07, 0.35, 0.95 for $\text{IκB} \alpha_{67-287} W$ from the kinetic m-values using the following equations:

\[
\begin{align*}
\alpha_{TSE1} &= -\frac{m_{12}}{m_{eq}} \\
\alpha_{TSE2} &= \frac{-m_{12} + m_{21}}{m_{eq}} \\
\alpha_{TSE3} &= \frac{-m_{12} + m_{21} + m_{34}}{m_{eq}}
\end{align*}
\]
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Table 2.3. Folding landscape of IκBα_{67-206}W mutants, continued

B. IκBα_{67-287}W mutants

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<th>ΔΔG_{TSE 2}</th>
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<tr>
<td>V203L</td>
<td>-0.2</td>
<td>-0.21</td>
<td>-0.42</td>
<td>-0.19</td>
<td>n/a(^b)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ΔΔG’s were calculated in comparison to WT IκBα_{67-206}W or IκBα_{67-287}W for using data from kinetics experiments. Φ-values were calculated from ΔΔG_{TSE}/ΔΔG_{eq}.

\(^b\) Phi-values were not calculated for mutants with ΔΔG_{eq} less than 0.5.
more apparent by plotting the average phi-value for each group against the degree of compaction along the reaction coordinate, $\alpha$ (Figure 2.8d). AR 3 and 4 again show folded character much earlier than AR 1 and 2, which are not folded until the folding reaction is nearly complete. This result recapitulates previous phi-value analyses on other four AR-containing proteins, p16 (Tang, Fersht et al. 2003) and myotrophin (Lowe and Itzhaki 2007). For p16, the majority of mutants have high phi-values; only a few mutants, clustered on ARs 1 and 2, have phi-values near 0. Myotrophin showed a similarly C-terminal-polarized transition state.

To uncover more detail, residue-specific phi-value analysis was performed on each of the TSEs for the $\zeta$B$\alpha_{67-206}$W mutants that had an overall $\text{DDG}_{eq} > 0.5 \text{kcal mol}^{-1}$ (Table 2.3). The phi-values revealed that residues L163, T164, and G194 had high phi-values in TSE1 (Figure 2.9a). These residues are all located in helix 2 (L163 and T164 in AR3 and G194 in helix 4). Thus, folding appears to initiate with only the interaction between the outer helices of AR3 and AR4. Although myotrophin, p16, and $\zeta$B$\alpha_{67-206}$W all have similar transition states with parts or all of the two C-terminal repeats folded, $\zeta$B$\alpha_{67-206}$W appears to have a much smaller folded region, at least in the first TSE, with only the outer helices of ARs 3 and 4 folded.

Moderate phi-values for N122 and A127 indicate that helix 2 in AR2 of $\zeta$B$\alpha_{67-206}$W folds in TSE2 (Figure 2.9b). Consistent with the Leffler analysis, it isn’t until TSE3, which is 95% as compact as the native state, that residues within helix 1 and the b-hairpins (Q111 and C186) appear to fold (Figure 2.9c). In fact, the C186P mutation in AR4 only has a phi-value near one in TSE3 indicating that all of AR4 is
Figure 2.9. Phi-values for (a) TSE 1, (b) TSE 2, and (c) TSE 3 are plotted on the structure of IκBα67-206W. The backbone of the protein is colored as in Figure 2.1. Mutated residues are shown in spheres and colored by Φ-value, with yellow for below 0.3, orange for 0.3 to 0.6, and red above 0.6, modifications performed in PyMol (DeLano 2002).
not folded at once. Indeed, the results presented here suggest that the two parts of the consensus represent different folding/stacking nuclei and that the outer helices (helix 2 of each repeat) fold/stack early in the folding pathway whereas the inner helices (helix 1) and b-hairpins fold/stack later. It is possible, then, that as was suggested in our earlier theoretical work (Ferreiro and Wolynes 2008), a single AR does not represent a single foldon and that further refinements of a general ARD folding mechanism will be required, in which other elements are treated as foldons rather than whole ARs.

3. Comparison of the 4AR and 6AR proteins

We had previously shown via equilibrium experiments that AR5-6 did not appear to contribute to the equilibrium stability of IkBα. Here we showed for three separate cases that mutations in the cooperative folding 4AR unit had the same effect in the full 6AR context. These results strongly suggest that the not only do AR5-6 not contribute to equilibrium stability, they also do not affect to the folding pathway of the cooperatively folding part of the IkBα ARD. This is in contrast to many other studies in which adding ARs to a cooperatively folding ARD enhances stability and sometimes alters the folding pathway (Tripp and Barrick 2004; Low, Weininger et al. 2007; Tripp and Barrick 2007). These results again highlight that the AR5-6 region of IkBα is weakly folded and will perhaps have unique characteristics as compared to repeats in other well-studied ankyrin repeat domain-containing proteins.
E. References


Chapter II, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in *Journal of Molecular Biology*.

Chapter III

Consequences of IkappaB alpha hydroxylation by the factor inhibiting HIF (FIH)
A. Introduction

The hypoxia-inducible transcription factors (HIFs) mediate the genomic response to oxygen deficiency (hypoxia) in multicellular organisms. These transcription factors are $\alpha/\beta$ heterodimers that bind DNA and activate transcription of over 70 target genes during cellular hypoxia (Semenza 2003). HIFs “sense” the level of oxygen by being modified by four oxygen-sensitive hydroxylases; three prolyl hydroxylases (PHDs) and one asparaginyl hydroxylase, factor inhibiting HIF-1 (FIH) (Schofield and Ratcliffe 2004). These hydroxylases are all members of the 2-oxoglutarate (2OG)–dependent dioxygenase superfamily and their activity depends on the cellular oxygen concentration. Under normoxia, the HIF-$\alpha$ subunit is hydroxylated by the PHDs leading to ubiquitination and rapid degradation. In addition, FIH-mediated hydroxylation regulates the transcriptional activity of the HIF-$\alpha$ proteins by inhibiting binding of the obligate CREB-binding protein (CBP)/p300 coactivator proteins to the HIF-$\alpha$ C-terminal transactivation domain (CAD), further repressing transcriptional activity (Hewitson, McNeill et al. 2002; Lando, Peet et al. 2002). At the onset of hypoxia, hydroxylase activity is greatly reduced, resulting in HIF protein accumulation and derepression of CAD activity, and consequently robust transcriptional activation.

Recent work has demonstrated that, in addition to hydroxylating HIF-$\alpha$, FIH can also hydroxylate the ankyrin domains of a wide range of proteins. Ankyrin repeat domains contain a consensus sequence that has been shown to bind to, and be a good substrate for, FIH (Cockman, Webb et al. 2009). Searches for alternative substrates of
FIH identified IκB, Notch family members and ASB4 (ankyrin repeat and SOCS box protein 4) as substrates of FIH (Cockman, Lancaster et al. 2006; Coleman, McDonough et al. 2007; Ferguson, Wu et al. 2007; Zheng, Linke et al. 2008; Cockman, Webb et al. 2009; Cockman, Webb et al. 2009). These intracellular proteins all contain ankyrin repeat domains (ARDs), and in each case the target asparagine residues lie within the ARD. Ankyrin repeat-containing proteins are found in all three phyla, and are present in some 6% of eukaryotic protein sequences (Mosavi, Cammett et al. 2004; Björklund, Ekman et al. 2006). Thus the consequences of FIH-dependent hydroxylation could be wide-ranging if modification of these alternate substrates has functional significance. Although these alternative substrates have been shown to be hydroxylated in vitro, in cultured cells, and in vivo, it remains difficult to establish functional consequences of the hydroxylation event in a rigorous manner.

Since hydroxylation of HIF-α potentiates its degradation, and the degradation rate of IκBα is a sensitive parameter in control of the NFκB signaling pathway (O'Dea, Barken et al. 2007; Ferreiro and Komives 2010), we were particularly interested in the possible functional role of hydroxylation in regulating the stability and/or degradation of IκBα. Indeed, human IκBα is hydroxylated by FIH at positions 210 and 244 (Figure 3.1A) with Asn 244 being the preferred site (Cockman, Lancaster et al. 2006). The two hydroxylated asparagines are at the interfaces between AR4-AR5 and AR5-AR6, which is the weakly-folded part of IκBα (Croy, Bergqvist et al. 2004) that folds on binding of NFκB (Figure 3.1A) (Truhlar, Torpey et al. 2006). In previous work, we showed that stabilization of AR5-AR6 in IκBα altered its intracellular half-life and had drastic functional consequences (Truhlar, Mathes et al. 2008). Given that
Figure 3.1. A) Structure of IκBα from the NFκB-IκBα complex showing the relative amide exchange in free IκBα vs. NFκB-bound IκBα. The red regions exchanged nearly all of their amides within 2 min, whereas the blue regions exchange less. Gold spheres mark the positions of the two hydroxylated asparagines, Asn 210 and Asn 244. B) Sequence of IκBα showing the two hydroxylated asparagines in bold-face type. Above the sequence is the secondary structural representation of each ankyrin repeat. The lines underneath the sequence depict the peptides generated upon pepsin cleavage during the amide exchange experiment that were analyzed. The solid lines indicate peptides for which amide exchange can be quantitatively determined whereas the dashed lines indicate peptides for which the amide exchange can only be qualitatively assessed.
hydroxylation has been shown to stabilize consensus ARD proteins, it is possible that hydroxylation might increase the stability of the IκBα ARD, and therefore influence its functional activity (Hardy, Prokes et al. 2009). Here we present a full biophysical characterization of FIH-hydroxylated IκBα, including binding of the hydroxylated protein to NFκB and proteasomal degradation rates.

B. Materials and methods

1. IκBα expression and purification

Human IκBα67-287 protein was expressed and purified as described previously (Truhlar, Mathes et al. 2008). Residues 67-287 comprise the entire ARD and the PEST sequence. This protein is truncated at the C-terminus, and is missing residues 288-317, but we have shown previously that it has identical folding and binding properties to the full-length ARD (Bergqvist, Croy et al. 2006). The final purification step was on a Superdex-75 gel filtration column (GE Healthcare) to ensure the absence of any aggregated species. The proteins were stored at 4°C and used within 3 days of gel filtration. Protein concentrations were determined by spectrophotometry, using extinction coefficient of 12,950 M⁻¹ cm⁻¹ for IκBα.

2. In vivo hydroxylation

In vivo hydroxylation was achieved by co-expressing IκBα behind the T7 promoter in a kanamycin resistant vector and Trx-6H-FIH (Linke, Hampton-Smith et al. 2007) behind the T7 promoter in an ampicillin resistant vector in BL21 (DE3) cells. Hydroxylated IκBα was purified from the co-expression system as described above.
Although some of the IκBα remained in a high molecular weight complex with FIH, monomeric, hydroxylated IκBα could be obtained after a final purification step on Superdex-75 gel filtration column.

3. Mass spectrometry

Hydroxylated IκBα samples were analyzed by liquid chromatography (LC)-MS with electrospray ionization. All nanospray ionization experiments were performed by using a QSTAR-XL hybrid mass spectrometer (ABSciex) interfaced to a nanoscale reversed-phase high-pressure liquid chromatograph (Tempo) using a 10 cm-180 ID glass capillary packed with 5-µm Magic C-4 beads (Michrom). Buffer A was 98% H2O, 2% ACN, 0.2% formic acid, and 0.005% TFA and buffer B was 100% ACN, 0.2% formic acid, and 0.005% TFA. Peptides were eluted from the C-4 column into the mass spectrometer using a linear gradient of 25–80% Buffer B over 60 min at 400 µl/min. LC-MS data were acquired in TOF scan mode (400-2000). The parameters for ESI-MS were: IS 2.3 KV; DP 65 V; CAD 5 V. The data were processed with Analyst 2.0 software using Bayesian Protein Reconstruction tool in the mass range 20,000-30,000 with step mass of 1 Da ans S/N threshold of 20.

4. Equilibrium folding experiments

Equilibrium folding experiments were performed on an Aviv 202 spectropolarimeter (Aviv Biomedical, Lakewood, NJ, USA) with a Hamilton Microlab 500 titrator (Hamilton, Reno, NV, USA). Urea (Fisher Scientific, Pittsburg, PA, USA) was specially purified by dissolving it at a nominal concentration of 8M in water, and then treating with AG 501-X8 (D) resin (BioRad Laboratories, Hercules, CA, USA)
for 1 hour to remove cyanate contaminants (Street, Courtemanche et al. 2008). Resin was filtered out with a 0.2µm filter and buffer salts were added to the purified urea. Urea concentrations were checked using refractometry (Pace 1986). Urea was used within 2 days of resin treatment to prevent re-accumulation of cyanate. A 1 cm fluorescence quartz cuvette containing 2.0 ml of 0.5–4 µM of the native protein in buffer (25 mM tris, 50 mM NaCl, 1 mM DTT, 0.5 mM EDTA, pH 7.5) and was titrated with denatured protein (7.3 – 8.4 M urea in buffer), in 30 to 40 injection steps. After each injection, samples were equilibrated with constant stirring at 80 rpm for 180s prior to data collection. The CD signal was collected at 225 nm, averaged 10 seconds, and the fluorescence signal was collected through a 320 cut-off filter with an excitation wavelength of 280nm, averaged over 2 to 5 seconds. Experiments were performed at 5°C unless otherwise stated.

Folding curves were fit to a two state folding model, assuming a linear dependence of the folding free energy on denaturant concentration (Pace 1986). The pre (native) and post (unfolded) transition baselines were treated as linearly dependent on denaturant concentration. The data was globally fit to equation 1, 

$$S_{obs} = (a_1 + p_1[Urea]) + (a_2 + p_2[Urea]) \exp(-\Delta G - m[Urea]/RT)) / (1 + \exp(-\Delta G - m[Urea]/RT)))$$  

Equation 1

where $S_{obs}$ is the observed signal, $p_1$ and $p_2$ are the pre and post transition baselines, $a_1$ and $a_2$ their corresponding y-intercepts; $\Delta G$ is the folding free energy in water and $m$ is the cooperativity parameter (m-value). The data were fit using a non-linear least square fitting algorithm in Kaleidagraph (Synergy Software, Reading, PA, USA).

5. Amide exchange experiments
Native state backbone amide exchange was measured as described previously (Ferreiro, Cervantes et al. 2007). To specifically probe the weakly-folded parts of the ARD, IκBα was incubated for 2 min in deuterated buffer at pH 7.4 and then the amide exchange was quenched by dilution into ice cold 0.1% TFA solution to bring the pH down to 2.2 and the temperature down to 0°C. The sample was immediately digested with an excess of immobilized pepsin and the digest mixture was frozen in liquid N₂. All samples were analyzed on the same day by MALDI-TOF mass spectrometry (Mandell, Falick et al. 1998). Eighteen peptides that cover 60% of the sequence were analyzed for both wild-type and hydroxylated IκBα. The centroids of the mass envelopes were measured and compared to undeuterated controls and corrected for back exchange as described previously (Croy, Bergqvist et al. 2004).

6. **Surface plasmon resonance binding experiments**

Sensorgrams were recorded on a Biacore 3000 instrument using streptavidin chips as described (Bergqvist, Croy et al. 2006). NFκB was biotinylated and immobilized as described; 150 RU, 250 RU, and 350 RU of NFκB (p50\textsubscript{248-321}/p65\textsubscript{190–321}) were immobilized. Wild type IκBα (0.02–1.6 nM) was injected for 5 min and dissociation was measured for 20 min at 25 °C at 50 μL/min. Regeneration was achieved by a 1 min pulse of 3 M urea in running buffer. Data were fit using the BiaEvaluation 2.0 software and plotted in Origin 7.0.

7. **Proteasome degradation experiments**

Proteasome degradation assays were performed basically as described (Truhlar, Mathes et al. 2008). IκBα (1 μM), freshly purified by size-exclusion
chromatography, was incubated with 100nM bovine 20S proteasome (a gift from G. Ghosh) for 0 min, 15 min, 30 min, 60 min, 90 min, or 120 min at 25 °C in 20 mM Tris, 200 mM NaCl, 10 mM MgCl₂, 1 mM DTT (pH 7.0). Degradation reactions were quenched by boiling with SDS-PAGE sample buffer. Intact IκBα was separated by SDS-PAGE (13% polyacrylamide gel) and visualized using Western blots probed with sc-847 (Santa Cruz Biotechnologies) followed by anti-rabbit HRP conjugate.

C. Results

1. Preparation and characterization of FIH-1-hydroxylated IκBα

Several different approaches to obtaining fully-hydroxylated IκBα were attempted, including in vitro incubation with either purified FIH or lysates from E. coli cells expressing FIH. The FIH and IκBα appeared to form a tight complex prior to hydroxylation, and only partial hydroxylation was observed under initial in vitro hydroxylation conditions tested (data not shown). Co-expression of the IκBα with FIH in E. coli finally resulted in sufficient yields of hydroxylated protein. Mass spectrometric analysis of the hydroxylated protein compared to unhydroxylated controls showed that the protein was fully hydroxylated with only one predominant peak at 24,427 Da after deconvolution of the multiply charged species, as compared to the unhydroxylated control, which had a mass of 24,394 Da. Others have shown that FIH hydroxylates IκBα at Asn 210 and Asn 244, but the hydroxylation at 244 occurs more readily (Cockman, Lancaster et al. 2006). Pepsin digestion of the hydroxylated protein gave four peptides spanning residues 203-221 (Figure 3.1). Analysis of these
**Figure 3.2.** MALDI-Tof mass spectra of the peptide corresponding to residues 202-221 (m/z 2028) from wild type IκBα (grey) and hydroxylated IκBα. Although quantitation is not possible due to the expected difference in ionization between the hydroxylated and unhydroxylated peptides, it is clear that a substantial amount of Asn 210 is hydroxylated.
peptides showed that at least 50% of the IκBα was hydroxylated also at Asn 210 (Figure 3.2).

2. Equilibrium unfolding experiments

The hydroxylated protein displayed high α-helical secondary structure content, as indicated by the far-UV circular dichroism (CD) spectrum, which was not different from the wild type (Figure 3.3A). To probe the stability of the FIH hydroxylated IκBα, titration experiments were performed to unfold the protein in urea monitoring the CD signal at 222 nm. Although IkBa is prone to aggregation and is not thermally denaturable, at pH 7.5, in 50 mM NaCl, 1mM DTT, 25 mM Tris-HCl buffer, the refolding upon chemical denaturation was >95% reversible, as shown previously (Ferreiro, Cervantes et al. 2007). The CD signal changed upon addition of denaturant and displayed a sharp transition, typical of a cooperatively folded unit, but with a sloping pre-transition baseline which we have previously assigned to the weakly-folded AR5-6 region (Ferreiro, Cervantes et al. 2007). Multiple experiments on the hydroxylated versus wild type IκBα were performed, however the equilibrium denaturation properties of the hydroxylated protein were not different from those of wild type IκBα (Figure 3.3B).

3. Amide hydrogen exchange experiments

Native state amide exchange monitored by MALDI-TOF mass spectrometry is a sensitive probe of the folded state of proteins in general, and is highly informative in IκBα because pepsin digestion after exchange produces fragments of similar
Figure 3.3. A) Circular dichroism (CD) spectra of wildtype (black) and hydroxylated (blue) IκBα. B) Equilibrium urea denaturation of IκBα (black) and hydroxylated IκBα (blue) at 3 mM total protein concentration at 5°C, (conditions described in Materials and Methods) followed by the CD signal at 225nm. The standard error of the mean from several experiments is also plotted. The data were fit assuming a sloping baseline giving for the wild type protein $\Delta G = -9.1 \pm 0.7$ kcal/mol, $m=2.6 \pm 0.2$ and for the hydroxylated protein $\Delta G = -9.3 \pm 0.5$ kcal/mol, $m=2.6 \pm 0.13$ kcal mol$^{-1}$ M$^{-1}$. 
secondary structure from each repeat that can be directly compared (Croy, Bergqvist et al. 2004). We have previously shown that mutation of only two residues in AR6 pre-fold the weakly-folded part, and this is most clearly shown by a dramatic decrease in amide exchange (a difference of some 5 deuterons) in AR5 and AR6 in this pre-folded mutant (Truhlar, Mathes et al. 2008). Figure 3.4 shows plots of the amide exchange of several peptides corresponding to regions of AR4-AR5. Comparison of the hydroxylated IκBα to the wild type protein shows no difference in amide exchange. This mass spectrometry experiment is particularly important because here the peptides corresponding to the hydroxylated protein are separated (by virtue of their difference in mass) from those of the unhydroxylated protein, even if they are present in the same mixture. Thus, this experiment allows us to compare the fully hydroxylated protein to the protein with only a hydroxyl at Asn 244 in the same experiment. It is clear from the plots in Figure 3.4 that again, no differences were observed in the “foldedness” of IκBα due to FIH hydroxylation. Although the peptide of mass 1767 from AR6 could not be analyzed quantitatively, this region also did not differ as assessed by qualitative comparisons of the mass envelopes.

4. Binding to NFκB by surface plasmon resonance

To probe whether the hydroxylation affects NFκB binding, surface plasmon resonance binding kinetics experiments were performed. As shown previously, IκBα binds extremely tightly to NFκB (Bergqvist, Croy et al. 2006). The binding experiments showed that hydroxylation had little, if any, effect on NFκB binding ($K_D$
Figure 3.4. Plots of the amide H/D exchange over five minutes into regions of IκBα or hydroxylated IκBα. In the case of the peptides covering the Asn 210, only the peptide of mass 2165 is presented, but all the peptides from this region gave the same results. For this peptide, three data sets are presented. Data for the unhydroxylated protein was obtained from a separate unhydroxylated sample as well as from the unhydroxylated protein present in the sample of hydroxylated protein.
of the wild type protein was 62 ±12 pM whereas that for the hydroxylated protein was 88 ±14 pM) (Figure 3.5).

5. Proteasomal degradation

We have previously shown that IκBα is rapidly degraded in vitro and in vivo by the 20S proteasome (Truhlar, Mathes et al. 2008). This process is ubiquitin independent, but rather depends on a degron in the C-terminal part of the protein that might be sensitive to FIH hydroxylation (Mathes, O'Dea et al. 2008). To probe whether FIH hydroxylation affected the degradation rate of IκBα by the 20S proteasome, similar in vitro experiments were performed. Figure 3.6 shows that the degradation of FIH-hydroxylated IκBα is not significantly different from that of wild type IκBα. Quantitation of the bands by densitometry gives a half-life of 15 min for both the hydroxylated and unhydroxylated protein.

D. Discussion

The NFκB signaling pathway is controlled by several inhibitor proteins, but IκBα is responsible for the rapid response after acute stress signals (Hoffmann, Levchenko et al. 2002). Properties of IκBα including its “foldedness”, intracellular half-life, and binding affinity for NFκB dimers are all exquisitely balanced in order to maximize pathway response and control (Ferreiro and Komives 2010). In addition, NFκB signaling has previously been shown to be induced by hypoxia although the mechanism of induction is not fully understood (Schmedtje, Ji et al. 1997; Zampetaki, Mitsialis et al. 2004). Previous reports indicated a slightly enhanced inhibitory action
Figure 3.5. Surface plasmon resonance (Biacore) binding kinetics of the interaction between NFκB and either wild type (A) or hydroxylated (B) IκBα was performed as previously described (Bergqvist, Croy et al. 2006). NFκB(p5248-321/p65190-321) was biotinylated at the N-terminus of p65 and immobilized on a streptavidin sensor chip. IκBα (0.12, 0.34, 0.56, 0.95, 1.56 nM) was the flowing analyte. The data were fit using a simple 1:1 binding model yielding for the wild type protein $k_a = 6 \times 10^6$ M$^{-1}$s$^{-1}$, $k_d = 2.3 \times 10^{-4}$, $R_{\text{MAX}} = 28.3$, $C^2 = 0.55$, and $K_D = 62 \pm 12$ pM and for the hydroxylated protein $k_a = 4 \times 10^6$ M$^{-1}$s$^{-1}$, $k_d = 2.8 \times 10^{-4}$, $R_{\text{MAX}} = 44.3$, $C^2 = 0.74$, and $K_D = 88 \pm 14$ pM.
Figure 3.6. Proteasome degradation experiments were performed as described in the Materials and Methods section. (A) Samples were taken at 0, 30, 60, 90, and 120 min and quenched by addition of gel loading buffer. The amount of remaining IκBα was analyzed by SDS Page and Western blotting by comparing the sample containing proteasome (top gel) with the sample that did not contain proteasome (bottom gel). (B) Densitometric quantitation of the gels shown in A confirms the similar degradation rates of IκBα (●) and hydroxylated IκBα (▲).
of hydroxylated IκBα (that would presumably be suppressed by hypoxia) but siRNA-mediated suppression of FIH actually reduced NFκB activity (Cockman, Lancaster et al. 2006). Thus, although a functional significance would make a lot of sense, the evidence was tenuous at best. In previous work, we have demonstrated that we can subtly perturb the “foldedness” of the IκBα ARD and consequently its various functions, including NFκB binding and intracellular half-life through the use of stabilizing mutations (Ferreiro, Cervantes et al. 2007; Truhlar, Mathes et al. 2008). Here we used the same experimental approaches to analyze the consequences of FIH hydroxylation on the “foldedness” and function of IκBα.

Others have shown that IκBα is hydroxylated by FIH at two asparagine residues, Asn 210 and 244, the latter of which is more readily hydroxylated (Cockman, Lancaster et al. 2006). In attempting to hydroxylate IκBα with FIH in vitro, we observed that a tight complex forms between the two proteins as has been seen for FIH and other ARD-containing proteins (Hardy, Prokes et al. 2009). This complex was so tight as to make it difficult to separate the IκBα from the FIH to obtain reasonable yields of fully hydroxylated protein from in vitro reactions. Instead, from co-expression, we were able to obtain monomeric hydroxylated IκBα in reasonable yields. Electrospray Q-TOF mass spectrometry of the intact hydroxylated IκBα showed a single peak with a mass approximately 32 Da higher than IκBα suggesting that the protein was 100% hydroxylated at two sites. Pepsin digestion followed by MALDI mass spectrometry, which is not quantitative and is expected to cause at least some fragmentation of the hydroxyl group, showed that at least 50% of
Asn 210 was hydroxylated. Taken together, these data strongly suggest that the protein is 100% hydroxylated at Asn 244 and at least 50% hydroxylated at Asn 210.

Equilibrium unfolding experiments showed equal stability of the FIH hydroxylated \( \text{IkB} \alpha \) and consistent with this result, native state amide H/D exchange to probe the backbone dynamics also showed no difference between unhydroxylated and hydroxylated \( \text{IkB} \alpha \) across all of the ankyrin repeats. Importantly, even though the protein may not have been completely hydroxylated, the hydroxylated and unhydroxylated protein fragments separate by mass and could be simultaneously analyzed from the same sample.

Previous indirect DNA binding activity (EMSA assays) of NF\( \kappa \)B from nuclear extracts had suggested addition of FIH reduced the binding of NF\( \kappa \)B (Cockman, Lancaster et al. 2006). We previously showed that EMSA assays do not accurately recapitulate the intracellular binding affinity of the NF\( \kappa \)B-IkB\( \alpha \) interaction, but that surface plasmon resonance with specifically biotinylated NF\( \kappa \)B does (Bergqvist, Croy et al. 2006). We therefore measured the binding affinity of the hydroxylated IkB\( \alpha \) in the same manner and compared it to the unhydroxylated protein. The results clearly showed that hydroxylation had minimal effects on the binding affinity.

Finally, we previously showed that the intracellular half-life of IkB\( \alpha \) is exquisitely sensitive to the “foldedness” of AR5 and AR6 (Truhlar, Mathes et al. 2008). We also showed that intracellular half-life of free IkB\( \alpha \) is short because of proteasomal degradation of IkB\( \alpha \) that is ubiquitin independent and likely involves some form of the 20S proteasome (Mathes, O’Dea et al. 2008). As few as two
mutations in AR6 dramatically altered both the intracellular half-life and the in vitro 
half-life in the presence of 20S proteasome preparations (Truhlar, Mathes et al. 2008). 
Since intracellular half-life is one of the sensitive parameters that controls NFκB 
signaling, we were anticipating that FIH hydroxylation would alter the proteasomal 
sensitivity of IκBα. Again, however, both proteins had the same rate of degradation by 
20S proteasomal preparations.

Thus, for every function we can rigorously and quantitatively measure in vitro, 
FIH hydroxylation of IκBα is inconsequential. It is possible that many of the ARD 
hydroxylations that have been observed are similarly inconsequential and that the 
consensus sequence that is being hydroxylated is an accidental substrate. On the other 
hand, we have shown that the FIH orthologue from Tribolium castaneum (red flour 
beetle) can also hydroxylate the Tribolium Notch analogue (Hampton-Smith and Peet 
2009). This implies that this “accidental” modification is highly conserved and may 
have some functional consequence other than alteration of stability or NFκB binding. 
FIH plays an important role in regulating metabolism, with the phenotype of the FIH 
knockout mice displaying elevated metabolism, including higher respiration and heart 
rate, as well as energy expenditure (Zhang, Fu et al. 2010). It is unclear at this stage 
whether this phenotype is mediated by the HIF or ankyrin repeat substrates, although 
there is no obvious disruption to NFκB signalling.

It is interesting that a similar conundrum also plagues asparagine 
hydroxylation in the EGF-like domains of most extracellular proteins. Here too, no 
evidence that hydroxylation alters the structure or function of the EGF-like domain-
containing proteins has been forthcoming. Yet, the beta asparty/ asparaginyl
hydroxylase knockout mice display a distinct developmental phenotype, which the
authors concluded is due to altered hydroxylation of Jagged and probably also its
receptor, Notch (Dinchuk, Focht et al. 2002). So, although the molecular
consequences of EGF hydroxylation are yet to be defined, it obviously has an
important physiological role. As mass spectrometry advances and more post-
translational modifications are found, it will likely become more and more common
that the functionally relevant substrates are few in a mix of unintentional ones. In
addition, the functional consequences of such modifications may require years of
experimentation to establish.

E. References

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Notch ankyrin repeat domain by factor inhibiting hypoxia-inducible factor." J


Chapter IV

Kinetics of coupled folding and binding of $\text{IκB}^\alpha$ to NFκB
A. Introduction

More and more intrinsically disordered proteins are being discovered with disordered or weakly folded regions or whole domains that fully fold upon association to their binding partners (Wright and Dyson 2009). There are many advantages to this arrangement. Because of their flexibility, these proteins often have many binding partners putting them at the center of protein interaction networks (Wright and Dyson 2009). This flexibility can allow the proteins to conform to the shape of a binding partner, increasing complementarily, such as with colicin and TolB (Wright and Dyson 2009). Additionally, the flexibility can allow the protein to adopt different structures for different binding partners, as is the case with p53, which has been observed in helical, β-strand, or extended structures with different binding partners (Oldfield, Meng et al. 2008).

Two limiting mechanisms should be considered for coupled folding and binding: induced folding and conformational selection (Wright and Dyson 2009). In the induced folding mechanism, the proteins associate first followed by a conformational change or folding event. The encounter complex of pKID and KIX was observed to be largely unstructured, suggesting that folding of pKID is induced by binding to KIX (Turjanski, Gutkind et al. 2008). Conformational selection occurs when the unfolded protein transiently adopts the folded structure and while the protein is folded, binding occurs. Interestingly, mutants of staphylococcal nuclease were found to bind using both mechanisms (Onitsuka, Kamikubo et al. 2008). It is likely
that most systems where coupled folding and binding are seen, the actual mechanism lies somewhere in between induced folding and conformational selection.

Coupled folding and binding can influence the binding kinetics as described by the fly-casting mechanism (Shoemaker, Portman et al. 2000). This theorizes that an unfolded protein binds faster than a well-structured protein because the unfolded protein has a larger capture radius than the folded protein. Once initial contact occurs, folding occurs concomitantly with binding, bringing the binding partners together into their final bound structure. Simulations on pKID showed that the binding rate could be reduced by increasing the amount of structure in unbound pKID, supporting a fly-casting process (Turjanski, Gutkind et al. 2008).

IκBα, the inhibitor of NFκB, is not fully folded when free in solution. The last two ankyrin repeats (AR) of IκBα have been shown to fully fold only when IκBα is bound to NFκB (Truhlar, Torpey et al. 2006). The weakly folded nature of AR5-6 serves as a switch between degradation mechanisms. When folded and bound to NFκB, IκBα undergoes signal-induced ubiquitin-dependant proteosomal degradation (Ghosh, May et al. 1998). When IκBα is free, the flexibility of AR5-6 and the PEST sequence following them exposes the protein to a signal- and ubiquitin-independent proteosomal degradation process (Mathes, O'Dea et al. 2008; Mathes, Wang et al. 2010. This switch between degradation mechanisms is accompanied by a large difference in the intracellular half-life of IκBα: free and NFκB-bound IκBα have half-lives of 10 minutes and 12 hours respectively (O'Dea, 2007 #14). The short half-life of free IκBα keeps cellular levels of the inhibitor low, which is essential for robust activation of NFκB.
IκBα has a high affinity for NFκB, resulting from an extremely slow
dissociation rate (Bergqvist, Croy et al. 2006). This has been shown to be due, in part,
to the coupled folding and binding of AR5-6 of IκBα. AR5-6 can be pre-folded by the
addition of two mutations in the consensus of AR6: Y254L and T257A (Truhlar,
Mathes et al. 2008); this double mutant binds to NFκB with lower affinity, due to
to faster dissociation (Truhlar, Mathes et al. 2008).

Additionally, IκBα has been shown to accelerate the dissociation of NFκB
from DNA (Bergqvist, Alverdi et al. 2009). Stabilization of IκBα decreases this effect,
and pre-folding AR5-6 decreases the effect further. Recently, a ternary IκBα-NFκB-
DNA complex was observed by NMR and stopped-flow fluorescence (Sue, Alverdi et
al. 2011). The chemical shifts of IκBα backbone resonances observed in these NMR
experiments suggested that AR5-6 and the PEST sequence of IκBα resemble the free
state where as AR1-4 resemble the bound state in this ternary complex. Together,
these results suggest a mechanism whereby AR1-4 of IκBα binds the NFκB-DNA
complex, and folding and binding of AR5-6 and the PEST sequence results in the
dissociation of DNA.

Here, we examine the coupled folding and binding of IκBα AR5-6 by using the
tryptophan reporter in AR6, W258. We show that there is a significant change in the
fluorescence of the tryptophan reporter on binding of IκBα to NFκB, and that the rate
of fluorescence change increases with NFκB concentration. We also show that pre-
folding AR5-6 causes a significant reduction in binding rate, supporting a fly-casting
mechanism in IκBα binding.
B. Materials and Methods

1. Expression and purification of proteins

IκBα<sub>67-287</sub> was expressed and purified as described previously (Truhlar, Mathes et al. 2008). Residues 67-287 comprise the entire ARD and the PEST sequence. This protein is truncated at the C-terminus, and is missing residues 288-317, but we have shown previously that it has identical folding and binding properties to the full-length ARD (Bergqvist, Croy et al. 2006; Bergqvist, Ghosh et al. 2008; Bergqvist, Alverdi et al. 2009). The final purification step was on a Superdex-75 gel filtration column (GE Healthcare) to ensure the absence of any aggregated species. The proteins were stored at 4°C and used within 3 days of gel filtration. Protein concentrations were determined by spectrophotometry, using extinction coefficient of 12,950 M<sup>−1</sup> cm<sup>−1</sup> for IκBα.

NFκB heterodimer of p50(248–350)/p65(190–321) with a hexahistadine tag on the N-terminus of p65 was expressed and purified as described previously (Sue, Cervantes et al. 2008). The final purification step was on a Superdex-75 gel filtration column (GE Healthcare) to ensure the absence of any aggregated species. The proteins were stored at 4°C and used within 7 days of gel filtration. Protein concentrations were determined by spectrophotometry, using extinction coefficient of 22,900 M<sup>−1</sup> cm<sup>−1</sup> for NFκB.

2. Fluorescence measurements

Equilibrium fluorescence spectra were recorded on a Horiba Fluorolog in a 1-cm quartz cuvette at 20°C. Emission spectra were recorded between 300 nm and 450
nm using an excitation wavelength of 293 nm with 3 nm slits for both excitation and emission.

Kinetic fluorescence binding experiments were performed on an Applied Photophysics Pi*-180 stopped-flow instrument (Applied Photophysics, Leatherhead, UK). Equal volumes of 0.5 µM IκBα and 0.1 to 2.5 µM NFκB in buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, pH 7.5) were rapidly mixed at 25°C, unless otherwise noted. Fluorescence was collected perpendicular to the 280 nm emission with a 320 nm cutoff filter over 10 to 30 seconds. An average of at least 9 traces was fit to a sum of exponential decays:

\[
\text{Signal} = c + \sum_i A_i \exp(-k_i t)
\]

where \( c \) is the final fluorescence value, \( A_i \) is the amplitudes of the change in fluorescence of each phase, and \( k_i \) the observed rate of folding of each phase. Fitting was performed with Profit (QuantumSoft, Uetikon am See, Switzerland).

3. Surface plasmon resonance binding experiments

Sensorgrams were recorded on a Biacore 3000 instrument using streptavidin chips as described (Bergqvist, Croy et al. 2006). NFκB was biotinylated and immobilized as described; 150 RU, 250 RU, and 350 RU of NFκB (p50\text{248-321/p65\text{190-321}}) were immobilized by way of an N-terminal biotin on the p65 to a strepavidin chip. Wild type IκBα (0.02–1.6 nM) was injected for 5 min and dissociation was measured for 20 min at 25 °C at 50 µL/min. Regeneration was achieved by a 1 min pulse of 3 M urea in running buffer. Data were fit using the BiaEvaluation 2.0 software and plotted in Origin 7.0.
Figure 4.1. Structure of NFκB-IκBα complex showing IκBα (blue), p65 dimerization domain and nuclear localization sequence region (red), and p50 dimerization domain (green) (PDB: 1NFI) (Jacobs and Harrison 1998). Tryptophan residues are shown in yellow. Modifications performed in PyMol (DeLano 2002).
C. RESULTS

1. Equilibrium fluorescence spectra of IκBα and NFκB

IκBα_{67-287} has a single tryptophan residue, W258, located just after helix 1 of AR6 (Figure 4.1). In the free protein, this region is highly solvent exposed due to the weakly folded nature of AR5-6 (Truhlar, Torpey et al. 2006). W258 remains relatively solvent exposed when IκBα is bound to the NFκB dimerization domain (Figure 4.1). Thus, any change in the fluorescence of W258 would likely come from folding of AR5-6 rather than from interaction with residues from NFκB.

NFκB p50(248–350)/p65(190–321) contains two tryptophan residues, one each in p50 and p65 (Figure 4.1). They are not near the interface with IκBα, and are in the well-folded dimerization domains, so we thought it unlikely their fluorescence would change significantly upon binding to IκBα.

The emission spectra for IκBα, NFκB, and the IκBα-NFκB complex were measured (Figure 4.2a), and the spectrum of free NFκB was subtracted in order to compare the spectrum of the complex with that of free IκBα (Figure 4.2b). Binding to NFκB produces a blue shift in the emission maximum from 353 nm to 344 nm. No change in the emission maximum was observed when free and IκBα-bound NFκB were compared (Figure 4.2c).

To confirm these results, we used a tryptophan-free mutant of IκBα (W258F). Addition of W258F to NFκB did not resulted in any changes in the fluorescence spectrum (Figure 4.2d). Surface plasmon resonance (SPR) experiments confirm that
Figure 4.2. Fluorescence equilibrium binding experiments. (A) Fluorescence spectra of 0.5 µM WT IκBα (cyan), 0.5 µM NFκB (red), and 0.5 µM WT IκBα-NFκB complex (green). (B) Comparison of free IκBα (cyan) and bound IκBα (black). The spectrum for bound IκBα was obtained by subtracting the spectrum of free NFκB from that of the IκBα-NFκB complex. (C) Comparison of free NFκB (red) and bound NFκB (purple). The spectrum for bound NFκB was obtained by subtracting the spectrum of free IκBα from that of the IκBα-NFκB complex. (D) Spectra for 0.5 µM W258F IκBα (cyan), 0.5 µM NFκB (red), and W258F IκBα-NFκB complex (green).
Figure 4.3. Surface plasmon resonance (Biacore) binding kinetics of the interaction between NFκB and either wild type (A) or W258F (B) IκBα. NFκB(p50\textsubscript{248-321}/p65\textsubscript{190-321}) was biotinylated at the N-terminus of p65 and immobilized on a streptavidin sensor chip. IκBα was the flowing analyte. The data were fit using a simple 1:1 binding model yielding for the wild type protein $k_a = 1.9 \times 10^6$ M\textsuperscript{-1}s\textsuperscript{-1}, $k_d = 3.8 \times 10^{-4}$, $\chi^2 = 1.84$, and $K_D = 2 \times 10^{-10}$ M and for the W258F mutant $k_a = 41 \times 10^6$ M\textsuperscript{-1}s\textsuperscript{-1}, $k_d = 9.5 \times 10^{-4}$, $\chi^2 = 2.14$, and $K_D = 9 \times 10^{-10}$ M.
this mutant binds to NFκB, though with a slightly lower affinity than WT IκBα (Figure 4.3).

2. Kinetics of IκBα binding to NFκB

The kinetics of IκBα binding to NFκB were measured by stopped-flow fluorescence. Upon complex formation, an increase in fluorescence was observed (Figure 4.4a). The concentration of IκBα was kept constant, and the concentration of NFκB was increased, and binding was measured at ratios of IκBα to NFκB of 1:0.5 to 1:5. Fluorescence traces at each ratio were fit with one to three exponentials. One major phase and two minor phases were observed, based on relative amplitudes. Of the two minor phases, the phase that was faster than the main phase was observed sporadically at near equal IκBα and NFκB concentrations and the slower phase was mainly observed with NFκB in excess. Neither minor phase showed a concentration dependence (Figure 4.5).

When the rate of the main phase was plotted against the concentration of NFκB, a linear increase in rate with respect to NFκB concentration was observed (Figure 4.5). Since it is known that the $k_d$ for dissociation of the IκBα-NFκB complex is extremely slow, the rate dependence on NFκB concentration was fit to the equation:

$$k_{obs} = k_d + k_a[NFκB]$$

where $k_{obs}$ is the observed binding rate, $k_d$ is the dissociation rate, and $k_a$ is the association rate. Consistent with the slow dissociation rate, the intercept was essentially 0. The association rate of $10 \pm 1 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ was measured at 25°C (Table
Figure 4.4. Stopped-flow binding traces showing change in fluorescence as a function of time (•) after mixing of 0.25 µM IκBα mutants and 0.125 µM (1:0.5), 0.25 (1:1), or 0.75 µM (1:3) NFκB at various temperatures. Traces were fit with 1, 2, or 3 exponentials; the best fit is shown (black lines). (A) WT IκBα at 25°C; (B) WT IκBα at 15°C; (C) WT IκBα at 5°C; (D) W258F IκBα at 5°C; and (E) Y254L/T257A IκBα at 25°C.
Figure 4.5. Observed binding rates at 25°C for WT IκBα (0.5 µM) as a function of NFκB concentration: main phase (red), fast phase (blue), and slow phase (green). The main phase was fit with a line (red line) with a slope of $10 \pm 1 \times 10^6$ M$^{-1}$ s$^{-1}$ and a y-intercept of $-0.6 \pm 0.9$ s$^{-1}$. 
Table 4.1. Effect of temperature on IκBα association kinetics.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$k_a$ (x 10$^6$ M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>20°C</td>
<td>7.2 ± 0.7</td>
</tr>
<tr>
<td>15°C</td>
<td>7.2 ± 1</td>
</tr>
<tr>
<td>10°C</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>5°C</td>
<td>4.0 ± 0.2</td>
</tr>
</tbody>
</table>

Association rate ($k_a$) was determined by plotting the effect of NFκB concentration on observed binding rate. The slope of the resulting linear dependence is the $k_a$. Reported errors are fit errors.
1). This is 10-fold faster than the association rate measured previously by SPR (Bergqvist, Croy et al. 2006; Truhlar, Mathes et al. 2008). Such a difference between stopped-flow and SPR rates has been previously observed (Iakoucheva, Walker et al. 2002; Bergqvist, Alverdi et al. 2009) and has been attributed to the immobilization required in SPR experiments. Additionally, faster rates observed in stopped-flow may be due to measuring the initial encounter complex between substrates rather than the final bound state. The previously reported $k_d$ is very slow (Bergqvist, Croy et al. 2006). In future work, we plan to expand the concentration range to more fully explore the linear dependence on NFκB concentration. Next, we examined the effect of temperature on the binding kinetics of IκBα-NFκB. As expected, the observed rate of the main phase increased with temperature, as did the association rate (Figure 4.6, Table 1). The slower phase was observed more prominently at lower temperatures and displayed decreasing fluorescence rather than the increasing fluorescence observed at 25°C (Figure 4.3b,c).

We also measured the formation of the IκBα-NFκB complex using the trp-free IκBα mutant, W258F. Binding traces for this mutant showed no significant signal change, similar to traces observed for mixing of NFκB with buffer (Figure 4.3d). This result confirmed that the fluorescence changes observed mixing wild-type (WT) IκBα with NFκB were due to changes in W258 and likely monitor the folding and binding of AR5-6.
Figure 4.6. Effect of temperature on binding kinetics of WT IκBα. (A) Observed binding rates (main phase) of WT IκBα (0.5 µM) as a function of NFκB concentration: 25°C (red), 20°C (blue), 15°C (green), 10°C (cyan), and 5°C (pink). A linear fit of the observed rates at each temperature is shown in the same color scheme. (B) Association rate, $k_a$, calculated from the slopes in (A), as a function of temperature. A linear fit of the rates is shown and has a slope of $2.6 \pm 0.5 \times 10^5 \, \text{°C}^{-1} \, \text{s}^{-1}$ and a y-intercept of $3.0 \pm 0.8 \times 10^6 \, \text{s}^{-1}$.
3. Effect of IκBα stabilization on binding kinetics

Taking advantage of the consensus sequence for stabile ARDs, we were able to produce several IκBα mutants in which different regions of the IκBα ARD were stabilized (Chapter 3). The effect of stabilizing different regions of the protein on the IκBα-NFκB binding kinetics was examined using various stabilized mutants. Q111G has the largest effect on stability, increasing the stability of IκBα ARD by 2.3 kcal mol\(^{-1}\) (Ferreiro, Cervantes et al. 2007). Smaller effects are seen for T164L (0.5 kcal mol\(^{-1}\) more stable)(Devries, Ferreiro et al. 2011), a double mutant of C186P and A220P (1.3 kcal mol\(^{-1}\) more stable) (Ferreiro, Cervantes et al. 2007; Truhlar, Mathes et al. 2008), and a double mutant of Y254L and T257A (0.5 kcal mol\(^{-1}\) more stable)(Truhlar, Mathes et al. 2008). The Y254L/T257A mutant was shown to be pre-folded according to amide H/D exchange, the observation of all of the cross peaks in the HSQC spectrum, and the observation that W258 showed a cooperative denaturation curve (Truhlar, Mathes et al. 2008).

The Q111G, T164L, and C186P/A220P mutants all showed similar binding kinetics and have similar association rates as WT IκBα (Figure 4.7, Table 2). This result agrees with previous SPR experiments that showed the Q111G and C186P/A220P mutants had similar binding kinetics as WT (Bergqvist, Alverdi et al. 2009).

Binding of the Y254L/T257A mutant to NFκB resulted in a decrease in fluorescence rather than an increase as observed for WT and other mutants; and the change in fluorescence was also significantly smaller (Figure 4.4d). This result is not unexpected given that the W258 fluorescence of the Y254L/T257A mutant was
Figure 4.7. Effect of mutation on binding kinetics of WT \(I\kappa B\alpha\). Observed binding rates (main phase) of \(I\kappa B\alpha\) (0.5 \(\mu\)M) as a function of NFkB concentration: WT (red), C186P/A220P (blue), Q111G (green), T164L (cyan), and Y254L/T257A (pink). A linear fit of the observed rates at for each mutant is shown in the same color scheme.
**Table 4.2.** Effect of mutation on IκBα association kinetics.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_a$ ( x $10^6$ M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>C186P/A220P</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Q111G</td>
<td>9.7 ± 0.5</td>
</tr>
<tr>
<td>T164L</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>Y254L/T257A</td>
<td>5.0 ± 0.6</td>
</tr>
</tbody>
</table>

Association rate ($k_a$) was determined by plotting the effect of NFκB concentration on observed binding rate. The slope of the resulting linear dependence is the $k_a$. Reported errors are fit errors.
previously shown to resemble the fluorescence of the NFκB-bound IκBα (Truhlar, Mathes et al. 2008). The observed kinetics of the Y254L/T257A mutant were slower than for WT, resulting in a 1.8 fold decrease in the association rate (Figure 4.7, Table 2). Previous SPR studies had shown that both the Y254L/T257A mutant and WT IκBα bound with the same association rate of $1.1 \times 10^6$ M$^{-1}$ s$^{-1}$ (Truhlar, Mathes et al. 2008). Here, we see a difference in association kinetics, and much faster association rates, around $10^7$ M$^{-1}$ s$^{-1}$, are measured. We surmise that stopped-flow measures the initial encounter complex formation whereas SPR measures the final bound state.

D. Discussion

1. W258 is a convenient probe of local foldedness and therefore coupled folding and binding

   We have previously shown that AR5-6 folds only on binding to NFκB (Truhlar, Torpey et al. 2006). The only natural tryptophan in IκBα, W258, is located in AR6 and can potentially report on the foldedness of AR5-6. Indeed, the W258 was previously shown to report on the “foldedness” of the Y254L/T257A mutant IκBα (Truhlar, Mathes et al. 2008). Here, we are able to show that the fluorescence of W258 changes upon association with NFκB in both equilibrium and kinetic experiments. The fluorescence of the W258 in NFκB-bound IκBα resembled that of the free Y254L/T257A mutant IκBα. Therefore, W258 can be used to report on coupled folding and binding.
2. Stabilizing mutations reveal that local stability of the weakly-folded region affects association rate, not global stability of the entire ARD

Previous SPR studies have shown that stabilization of IκBα results in decreased binding affinity for NFκB (Truhlar, Mathes et al. 2008). Here, we used stopped-flow techniques to examine the effect of stabilization on the coupled folding and binding of stabilizing mutations in the IκBα ARD. The binding kinetics of four proteins with were examined and compared with the WT kinetics. Q111G, T164L, C186P/A220P, and Y254L/T257A all increased the global stability of IκBα, as reported by urea denaturation experiments (Ferreiro, Cervantes et al. 2007; Truhlar, Mathes et al. 2008; Devries, Ferreiro et al. 2011). Of the stabilized mutants, only the Y254L/T257A mutation results in the pre-folding of AR5-6, by specifically increasing the local stability in these repeats (Truhlar, Mathes et al. 2008).

Of these four stabilized proteins, Q111G, T164L, and C186P/A220P displayed similar binding kinetics to WT IκBα. Though not the most stabilized mutant, the Y254L/T257A mutant IκBα was the only protein with altered binding kinetics, slowing the binding. This suggests that the local stability in AR5-6, rather than global stability of the ARD is important in binding to NFκB.

Consistent with the pre-folded structure of the Y254L/T257A mutant, binding to NFκB actually resulted in a decrease in W258 fluorescence, rather than an increase as is seen for WT and the other mutants. Additionally, the change in fluorescence is much smaller than in for the other IκBα mutants. We can only speculate on the cause of these differences, but it may be that the Y254L/T257A mutant is close to the bound
conformation and merely undergoes a subtle conformational change of AR5-6 upon binding.

We measured a $k_a$ of $10^7$ M$^{-1}$ s$^{-1}$, 10-fold faster than previous SPR measurements. This difference has previously been observed between stopped-flow and SPR experiments (Iakoucheva, Walker et al. 2002; Bergqvist, Alverdi et al. 2009), and is mainly attributed to the fact that SPR experiments measure approach to pseudo flowing equilibrium, which reflects the final bound state whereas the stopped flow measures the initial encounter. That said, the overall $K_D$ measured by both techniques is usually similar. We were unable to calculate $K_D$ because we could not accurately determine the dissociation rate, $k_d$. However, for our purposes, it was more important to compare the association rates of several mutants. By both SPR (Bergqvist, Alverdi et al. 2009) and stopped-flow, both the C186P/A220P and Q111G mutants have similar association kinetics to WT IκBα. This is not the case for the Y254L/T257A mutant, for which we observe a 1.8-fold decrease in $k_a$ even though this difference was not observed in the SPR experiments. It is likely that the initial encounter complexes with NFκB are formed at different rates, but the composite rate of formation of the final bound complex is the same for the Y254L/T257A mutant and the WT IκBα.

3. Fly-casting

The fly-casting mechanism is an important theory regarding the coupled folding and binding of intrinsically disordered proteins (Shoemaker, Portman et al. 2000). The fly-casting theory explains that an unfolded protein has a greater capture radius than a well-folded protein, increasing the likelihood that the unfolded protein
will encounter its binding partner. This, in turn, increases the binding rate for the unfolded protein up to 1.6 times the rate of the completely folded protein.

AR5-6 of IκBα are weakly folded in free state and only fully fold upon binding to NFκB, thus a fly-casting binding mechanism is possible, though it has not previously been observed. Here, we measured the binding kinetics for a variety of stabilized IκBα mutants. The majority of mutants showed no apparent effect on the binding kinetics of IκBα, despite stabilization of the ARD from 0.5 to 2.3 kcal mol$^{-1}$. Only one mutant, Y254L/T257A, displayed altered binding kinetics, decreasing the $k_a$ by a factor of 1.8. The Y254L/T257A mutation has a moderate effect on stability, but more importantly, is fully folded in the free state. The fact that only this “pre-folded” mutant showed a decreased association rate strongly suggests a fly-casting mechanism.

Though the binding kinetics of Y254L/T257A were previously studied by SPR, no difference in association rate was observed between it and WT (Truhlar, Mathes et al. 2008). Here, we are able to observe a difference because the stopped-flow allows measurement of the initial encounter complex, rather than the final bound complex. For Y254L/T257A, the initial encounter complex forms more slowly, but is likely compensated for by faster final complex formation resulting in identical overall binding rates by SPR. Therefore, we suggest that the weakly folded nature of AR5-6 increases the rate of initial complex formation, but the formation of the final bound complex is limited by the folding of AR5-6.
E. References


Sue, S. C., V. Alverdi, et al. (2011). "Detection of a ternary complex of NF-{kappa}B and I{kappa}B{alpha} with DNA provides insights into how
I{\kappa}\text{B}{\alpha} removes NF-I{\kappa}\text{B} from transcription sites.\textcolor{red}{Proc Natl Acad Sci U S A 108}(4): 1367-72.


Chapter V

Conclusions and future work
In this work, I explored the folding and binding kinetics of IκBα using various biophysical techniques. In Chapter 2, I examined the folding kinetics of free IκBα using stopped-flow kinetics and analyzed the effect of consensus mutation on the folding kinetics. Through the use of phi-value analysis, I defined the folding unit for IκBα. We are the first to show that the folding unit is not the full ankyrin repeat (AR), but that two outer helices stack up first followed later by the inner helices of the same repeats. In a final step, the β-hairpins and remaining repeats then fold. Also, by through examination of the effect of mutation on the folding kinetics, we showed that each residue in the AR consensus contributes to the stability individually. Thus, the consensus sequence provides a powerful tool for probing how AR proteins are able to do what they do by providing a potentially infinite scale of stabilizing mutants. Finally, we demonstrated that the full ARD of IκBα folds by the same mechanism as the truncated AR1-4 construct, showing that ARs 5 and 6 do not contribute to the folding of free IκBα.

I measured the effect of asparagine hydroxylation on the folding and binding of IκBα using equilibrium denaturation, H/D exchange, and SPR in Chapter 3. We very accurately measured the folding, flexibility, and binding of WT and hydroxylated IκBα and saw that none of these properties were affected by hydroxyl modification.

In Chapter 4, I investigated the coupled folding and binding of IκBα using stopped-flow kinetics and the effect of mutation on this process. I showed by monitoring the fluorescence of a tryptophan in AR6 of IκBα that stabilization of the ARD by consensus mutation alone has no effect on the binding kinetics within the error of the experiment. Only by pre-folding AR5-6 did the binding kinetics change, as
the pre-folded mutant associated with NFκB more slowly than the WT IκBα. This showed that the weakly-folded nature of AR5-6 is necessary for the rapid binding of IκBα to NFκB. This result suggests a fly-casting mechanism where the capture radius of IκBα is expanded by the weakly folded repeats resulting in faster association kinetics.

The work in Chapter 4 will be expanded in the future. To further explore the linear correlation between binding rate and NFκB concentration, we will measure binding kinetics at a broader concentration range. Additionally, we will compare the binding kinetics of the pre-folded Y254L/T257A mutant to the WT at a variety of temperatures and in the presence of mild denaturant. We predict that the addition of mild denaturant, which result in increased flexibility, will increase the binding kinetics for the Y254L/T257A mutant.