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Author Manuscript

Adv Exp Med Biol. Author manuscript; available in PMC 2013 March 20.

Published in final edited form as:

Adv Exp Med Biol. 2012 ; 725: 74–85. doi:10.1007/978-1-4614-0659-4_5.

Consequences of Fuzziness in the NF-κB/IκB α Interaction

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Abstract

This chapter provides a short review of various biophysical experiments that have been applied to the inhibitor of kappa B, IκB α , and its binding partner, nuclear factor kappa B, or NF-κB. The picture that emerges from amide hydrogen/deuterium exchange, NMR, and binding kinetics experiments is one in which parts of both proteins are “fuzzy” in the free-state, and some parts remain “fuzzy” in the NF-κB•IκB α complex. The NF-κB family of transcription factors responds to inflammatory cytokines with rapid transcriptional activation, in which NF-κB enters the nucleus and binds DNA. Just as rapidly as transcription is activated, it is subsequently repressed by newly synthesized IκB α that also enters the nucleus and removes NF-κB from the DNA. Because IκB α is an ankyrin repeat protein, it’s “fuzziness” can be controlled by mutagenesis to stabilized the folded state. Experimental comparison with such stabilized mutants helps provide evidence that much of the system control depends on the “fuzziness” of IκB α .

Introduction

The nuclear factor κB (NF-κB) pathway transduces extra-cellular signals from various receptors to regulate patterns of gene expression ¹. Although originally discovered in B-cells because it strongly activates the immunoglobulin kappa-chain gene expression ², the pathway is ubiquitous, and has been implicated in a variety of cellular functions such as cell growth, proliferation, apoptosis, and stress responses and is missregulated in numerous diseases ^{3,4}. The family of NF-κB proteins includes p65 (RelA), RelB, c-Rel, p50, and p52 subunits, which form homo- and heterodimers ³ (Figure 1A). The most prevalent form in most cell types is a p50/p65 heterodimer, and the crystal structure of this form bound to a canonical kB DNA sequence has been solved ⁵ (Figure 1B). The inhibitors of NF-κB activity, IκBs, include isoforms IκB α , IκB β , and IκB ϵ , which block the nuclear localization and transcriptional activity of p65 and c- Rel-containing NF-κB dimers ⁶ and others that act in different pathways such as IκB δ ⁷ (Figure 1A). In resting cells, approximately 100,000 NF-κB dimers are nearly all bound to IκBs which keep the NF-κB in the cytoplasm by sequestering the NF-κB nuclear localization signal (NLS) ^{8,9}. The way in which the IκB α binds NF-κB was revealed from crystal structures of the NF-κB•IκB α ^{10,11}.

When a cell receives an extracellular signal such as a viral insult or cytokine, extracellular receptors activate the assembly of the IκB kinase (IKK), which in turn phosphorylates the N-terminal signal response domain of NF-κB-bound IκB α , leading to subsequent ubiquitination and degradation of the IκB α by the proteasome ¹². NF-κB dimers then translocate to the nucleus, bind DNA, and regulate transcription of numerous NF-κB target genes ¹³. NF-κB activated genes show widely varying transcription levels, activation kinetics, and post-induction repression, but how this single system results in so many

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different transcription effects is not well understood^{14,15}. The gene coding for IκBα is one of the strongly NF-κB-activated genes^{16–18}. When NF-κB transcription is activated, the resulting newly synthesized IκBα translocates to the nucleus, binds to NF-κB, and the complex is exported from the nucleus^{1,19}.

Free IκBα is rapidly degraded by a proteasome-dependent but ubiquitin-dependent mechanism, with a half-life less than 10 min. On the other hand, NF-κB-bound IκBα is incredibly stable, with an intracellular half-life of many hours consistent with the binding constant of the complex, of 40 pM^{9,20}. The IκBα in complex with NF-κB is only degraded if it is first phosphorylated, then ubiquitinated, and finally degraded by the proteasome in a ubiquitin-dependent fashion resulting in free, active NF-κB. A wealth of experimental data now suggests that the various functions of IκBα depend the partially folded character or “fuzziness” of parts of NF-κB and IκBα. These functions include the rapid degradation of the free protein, its tight binding to NF-κB, and its ability to dissociate NF-κB from the DNA. This review will briefly summarize the evidence for “fuzziness” and show the functional consequences of “fuzziness” in the NF-κB signaling system.

EXPERIMENTAL EVIDENCE OF IκBα “FUZZINESS”

NMR evidence of IκBα “fuzziness”

NMR studies of the entire 6-AR ARD of IκBα, residues 67–287, revealed that most of the cross peaks for AR5 and AR6 were missing, which likely indicated conformational exchange processes (CF Cervantes, unpublished data). In contrast, all of the resonances could be assigned for the 4-AR fragment containing residues 67–206²¹. When the chemical shift values for these residues were compared to those of the NF-κB-bound IκBα(67–287), they were nearly identical indicating that the structure of this part of IκBα is nearly identical in the free and bound states²². In addition, backbone dynamics experiments indicated that this part of the IκBα ARD is rigidly structured^{21,22}. Residual dipolar coupling (RDC) measurements were also performed on this fragment. RDCs predicted from the crystal structure of this part of IκBα bound to NF-κB did not agree well with the measured values and we surmised that this might be due to they report on motions from microseconds to hundreds of milliseconds (Figure 2A)²¹. Indeed, RDCs computed for an ensemble of structures of the IκBα(67–206) generated from accelerated molecular dynamics simulations agreed much better with the experimental data (Figure 2B). Thus, RDC measurements combined with AMD simulations might more realistically represent the solution ensemble in all its “fuzziness”.

Amide hydrogen/deuterium exchange of the IκBα ARD

Full-length IκBα is composed of three regions; an N-terminal signal response region of ~70 amino acids, where phosphorylation and ubiquitination occur, an ankyrin repeat domain (ARD) of ~220 amino acids, and a C-terminal PEST sequence that extends from residues 275–317^{10,11}. The NF-κB binding activity is localized to the ARD and PEST regions, for which high resolution crystal structures were obtained only when in complex with NF-κB. Sequence analyses predict intrinsic disorder in both the N-terminal domain and the PEST region of IκBα as well as in a good portion of the ARD (Figure 1A)²³. IκBα has resisted all attempts to crystallize it in the unbound state, and its biophysical behavior is consistent with a native state that does not adopt a unique compact fold²⁴. It is interesting to note the qualitative agreement between the the predicted disorder (Figure 3A) and the native state amide exchange (plotted as percent exchanged at 2 min) (Figure 3B).

Both I κ B α and NF- κ B fold on binding

The NF- κ B NLS folds on binding to I κ B α

NF- κ B family members such as the canonical member of the family, RelA, contain three main domains, the N-terminal domain, the dimerization domain, and the transactivation domain. I κ B α binds primarily to the dimerization domain whereas DNA binds in between the N-terminal and dimerization domains (Figure 1A). The crystal structure of the NF- κ B•I κ B α complex shows that I κ B α binds to NF- κ B in a head-to-tail fashion and the small sequence of NF- κ B containing the nuclear localization signal (NLS) forms two short helices that lay over the top of AR1 of I κ B α (Figure 1B)^{10,11}. The NLS connects the dimerization domain to the transactivation domain in the full length NF- κ B(p65) protein. In between the two helices is the KRKR sequence, which constitutes the minimal NLS¹¹. Theoretical studies of the binding of the NLS polypeptide (residues 291–325) of NF- κ B(p65) to I κ B α also suggested that this segment of NF- κ B folds on binding to I κ B α ²⁵. NMR heteronuclear single quantum coherence spectra of the NLS (residues 289–321 of NF- κ B (p65) clearly show that in the free-state the chemical shifts of the NLS backbone NHs are not well-dispersed and are mostly at random coil chemical shift values. In contrast, when bound to I κ B α , the chemical shifts are well-dispersed and at values expected for helical structure (Figure 4) (C.F. Cervantes, submitted). It has been experimentally observed that this segment binds with a 1 μ M K_D to I κ B α , and with a large $\Delta C_{P,obs}$ for I κ B α binding to this NLS segment (-1.30 ± 0.03 kcal mol⁻¹ K⁻¹) that could not be accounted for by burial of polar and non polar surface area calculations derived from the crystal structures^{26–28}. Thus, the thermodynamic signatures of the binding interaction cannot be accounted for by merely docking the individual static structures, and larger structural rearrangements must be implicated, as is often observed for protein-DNA interactions²⁹. Chemical shift values in the random coil region are a good indicator of lack of persistent structure in the free NLS, which must, therefore, be more “fuzzy” in the free-state than in the bound state where persistent helical structure is observed. The “head” of I κ B α (ARs 1–3) appears to be folded based on H/D exchange experiments, and the “tail” of NF- κ B (the NLS polypeptide) folds upon binding to it.

I κ B α folds on binding to NF- κ B

Native state amide H/D exchange experiments revealed that the fifth and sixth ARs exchange all of their amides within 2 minutes whereas the β -hairpins of AR2 and AR3 were remarkably resistant to exchange. (Figure 5)³⁰. The decrease in the number of exchanging amides could not be accounted for just by interface protection suggesting that I κ B α undergoes a folding transition upon binding. Amide exchange is an interesting probe of “fuzziness”. Although the rate of amide exchange depends on many factors that often cannot be teased apart, it can reliably report on relative differences. Thus, it is possible to compare the β -turns of each AR relative to one another as we did for the free protein, and it is also possible to compare the β -turn of a particular AR in the free vs. NF- κ B-bound state. In this latter case, one must consider the decrease in amide exchange due to decreased solvent accessibility at the protein-protein interface. In the case of I κ B α , we compared the solvent accessible surface areas calculated from the crystal structure of the NF- κ B•I κ B α complex to the results from amide exchange. These comparisons revealed that whereas the difference in exchange between bound and free was some 10 amides, the solvent accessible surface area was expected to change only by 2–3 amides (Figure 5)³¹. The large difference in amide exchange upon binding should therefore be attributed to differences in protein dynamics or “fuzziness” of the free protein that is lost upon binding.

REMAINING FUZZINESS IN THE NF-κB•IκBa COMPLEX

At the other end of the IκBa ARD, deletion of the PEST sequence (residues 276–287) reduces the NF-κB binding by some 5 kcal/mol³². Taken together, the binding affinity losses due to deletion at the ends of the interface are more than enough to account for the entire binding energy of complex formation. Interestingly, the PEST region does not become completely ordered upon binding to NF-κB according to high resolution NMR spectroscopy data³³. The native state of the NF-κB•IκBa complex thus retains regions with highly dynamic character.

NMR experiments on the NF-κB•IκBa complex revealed another interesting feature. Although amide exchange was very low in AR2 and AR3, NMR relaxation experiments on IκBa(67–206) indicated that the backbone of AR3 was more dynamic than was observed for the other ARs²¹. By finding the best AMD acceleration that matched the experimental RDCs, we were able to obtain a “picture” of the ensemble of structures that represents the solution state of the first four ARs of the IκBa ARD (Figure 6). It is clear that the variable loop connecting AR3 to AR4 is more “fuzzy” and the NMR data also indicated conformational exchange dynamics on the msec to msec time scale in the β-loop and helix of AR3 (colored red in Figure 6)²¹. NMR experiments further revealed that the dynamics observed in AR3 of free IκBa become even more excentuated upon binding to NF-κB. In the complex, many of the cross peaks for AR3 are not observed indicating strong conformational exchange to a range of chemical shift values making the peaks so broad as to become unobservable under the conditions of the experiment²². These results strongly support the idea that while some parts of IκBa (AR5 and AR6) become less “fuzzy” in the complex, other parts become more “fuzzy”.

FUNCTIONAL CONSEQUENCES OF FUZZINESS IN THE NF-κB•IκBa COMPLEX

Tight binding to multiple partners

NF-κB is a family of homo and heterodimeric molecules made of at least five different proteins. While the most prevalent form in many cell types is NF-κB(p50/p65), under certain conditions, the homodimeric form NF-κB(p65/p65) also becomes prevalent³⁴. The structure of this form of NF-κB bound to IκBβ has been solved, and it is remarkably similar to the structure of NF-κB(p50/p65) bound to IκBa³⁵. This is despite the fact that the sequences are not that similar. Kinetic and thermodynamic measurements of binding affinity show that IκBa binds to NF-κB(p50/p65) with nearly the same affinity as to NF-κB(p65/p65)⁹.

“Fuzziness” determines the degradation rate of IκBa

Free IκBa, which is marginally stable²⁴, has a very short intracellular half-life of less than 10 minutes^{20,36}. This rapid degradation rate depends in part on the presence of the C-terminal PEST sequence^{37–39}. The degradation of the free protein appears to be independent of ubiquitylation, since all of the Lys residues in IκBa can be mutated without changing the degradation rate of the free protein³⁹. In addition, although free IκBa can be phosphorylated and ubiquitylated, its degradation rate is not different in IKK–/– cells indicating that ubiquitin-independent degradation is the primary route for free IκBa²⁰.

To further probe how “fuzziness” was related to free IκBa degradation rate, we prepared a mutant in which two residues in AR6 were mutated to more commonly-found residues at those positions in other AR sequences. Taking advantage of the single tryptophan residue in IκBa at position 258, we showed that in the wild type protein, W258 did not show a

cooperative transition whereas in the mutant it did (Figure 7A, B). This is a strong indication that AR6 is not part of the cooperatively folding ARD in wild type I κ B α , but the mutations stabilize AR6 so that now the fluorescence signal from W258 follows the major cooperative transition (Figure 7B). Importantly, the Y254L, T257A mutant I κ B α is degraded more slowly than wild-type I κ B α both *in vitro* by the 20S proteasome and *in vivo* suggesting that in addition to the PEST sequence, the “fuzzy” AR6 of I κ B α is important for rapid ubiquitin-independent degradation (Figure 7C)⁴⁰. In contrast to the marginally-stable free I κ B α , the I κ B α •NF- κ B complex has a very long intracellular half-life of the complex, which is completely stable in the absence of I κ B kinase (IKK) phosphorylation and subsequent ubiquitylation (>12 hrs). Thus, I κ B α “fuzziness”, controlled by binding to NF- κ B, switches its degradation mechanisms³⁹.

“Fuzziness” is important for rapid signal repression by I κ B α

A key feature of the NF- κ B negative feedback is the rapidity with which the transcriptional activation is subsequently repressed¹. Rapid post-induction repression is partly explained by the fact that the gene for I κ B α is strongly induced by NF- κ B, so activation of NF- κ B immediately produces newly synthesized I κ B α . However, the new I κ B α must still escape proteasome degradation, enter the nucleus, and compete for binding to NF- κ B with the very large number of κ B sites in the DNA. We recently discovered an intriguing kinetic phenomenon in which I κ B α is able to markedly increase the rate of dissociation of NF- κ B from the DNA⁴¹. The phenomenon was initially discovered by flowing nanomolar concentrations of I κ B α over the NF- κ B•DNA complex in a co-injection step in an SPR experiment (Figure 8A). I κ B α is remarkably efficient at increasing the dissociation rate (k_d) of NF- κ B from the DNA; the apparent second order rate constant for the I κ B α -mediated dissociation is 10^6 M⁻¹ s⁻¹ (Figure 8B). Several mutant forms of I κ B α were also tested for their ability to mediate dissociation of NF- κ B from the DNA. The mutations had a variety of effects on NF- κ B binding, some bound with the same affinity, and some showed decreased affinity, up to 100-fold. However, all of the thermodynamically stabilized mutants, even the ones that bound with the same affinity, were less able to mediate dissociation of NF- κ B from the DNA (Figure 8C)⁴¹. Thus, an important function of the “fuzzy” AR6 in I κ B α may be to facilitate dissociation of NF- κ B from the DNA to rapidly repress post-induction transcriptional activation.

CONCLUDING REMARKS AND PERSPECTIVES

The NF- κ B signaling regulates many genes, and therefore is highly controlled. Biophysical experiments, including amide H/D exchange and NMR reveal that parts of both proteins are “fuzzy”. From these experiments, we see that “fuzziness” comes in many flavors, and only some parts of each protein are “fuzzy”. In addition, the “fuzziness” of I κ B α is reduced in some regions and increased in other regions upon binding to NF- κ B. More importantly, we have used biochemical experiments to show that “fuzziness” in I κ B α provides kinetic control of dynamic regulatory processes, including its degradation through Ub-dependent and independent pathways and competition with DNA for NF- κ B.

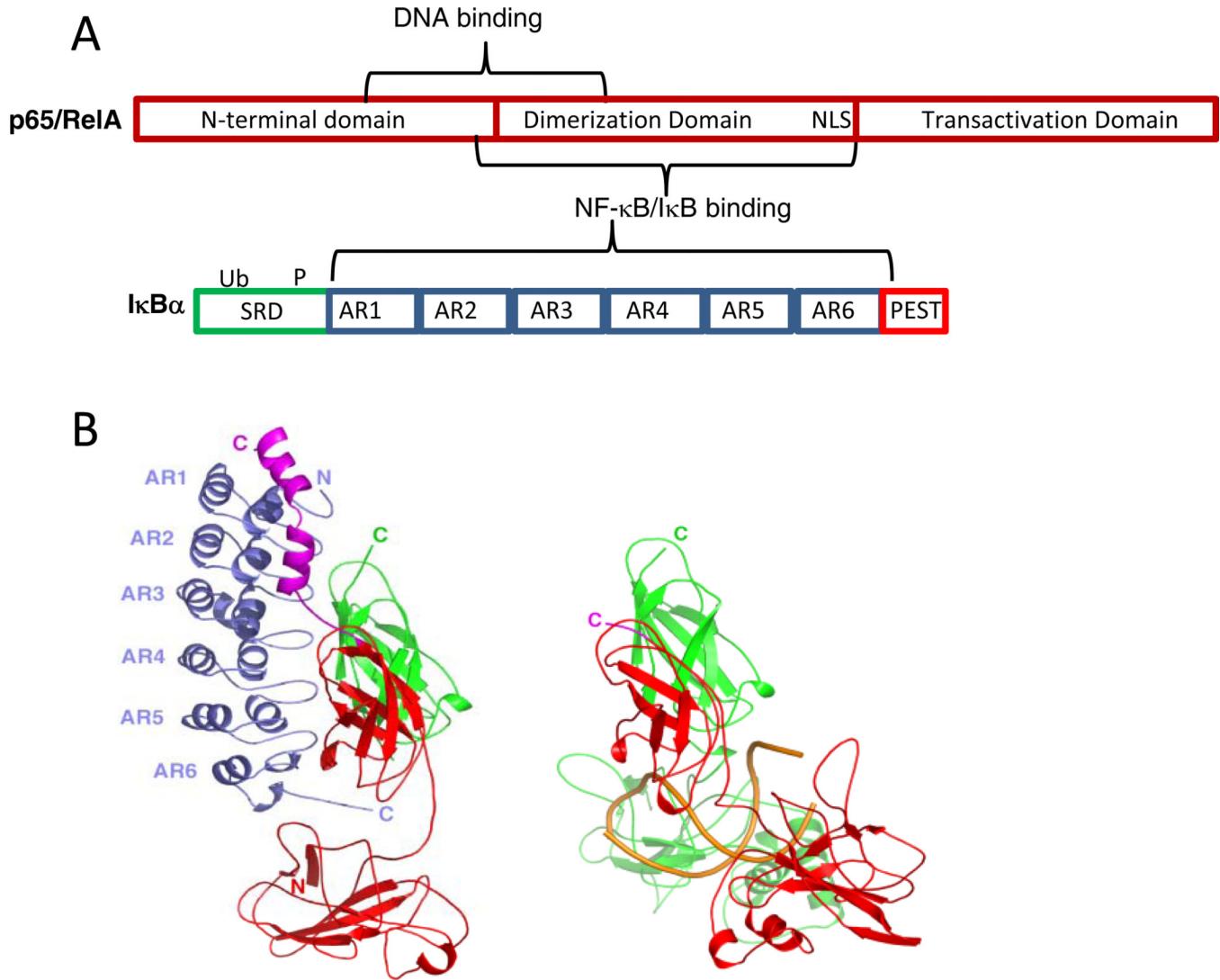
Abbreviations

I κ B	inhibitor of kappa B proteins
NF- κ B	nuclear factor kappa B
AR	ankyrin repeat
ARD	ankyrin repeat domain.

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**Figure 1.**

(A) Schematic diagram of NF- κ B(p65) one of the most abundant NF- κ B family members in the cell and of I κ B α , the key member of the inhibitor family. (B) LEFT: The crystal structure of I κ B α (blue) bound to NF- κ B (p50, green; p65, red)¹¹. RIGHT: The crystal structure of NF- κ B (p50, green; p65, red) bound to κ B site DNA (gold)⁵. (Figure prepared using PyMOL⁴²).

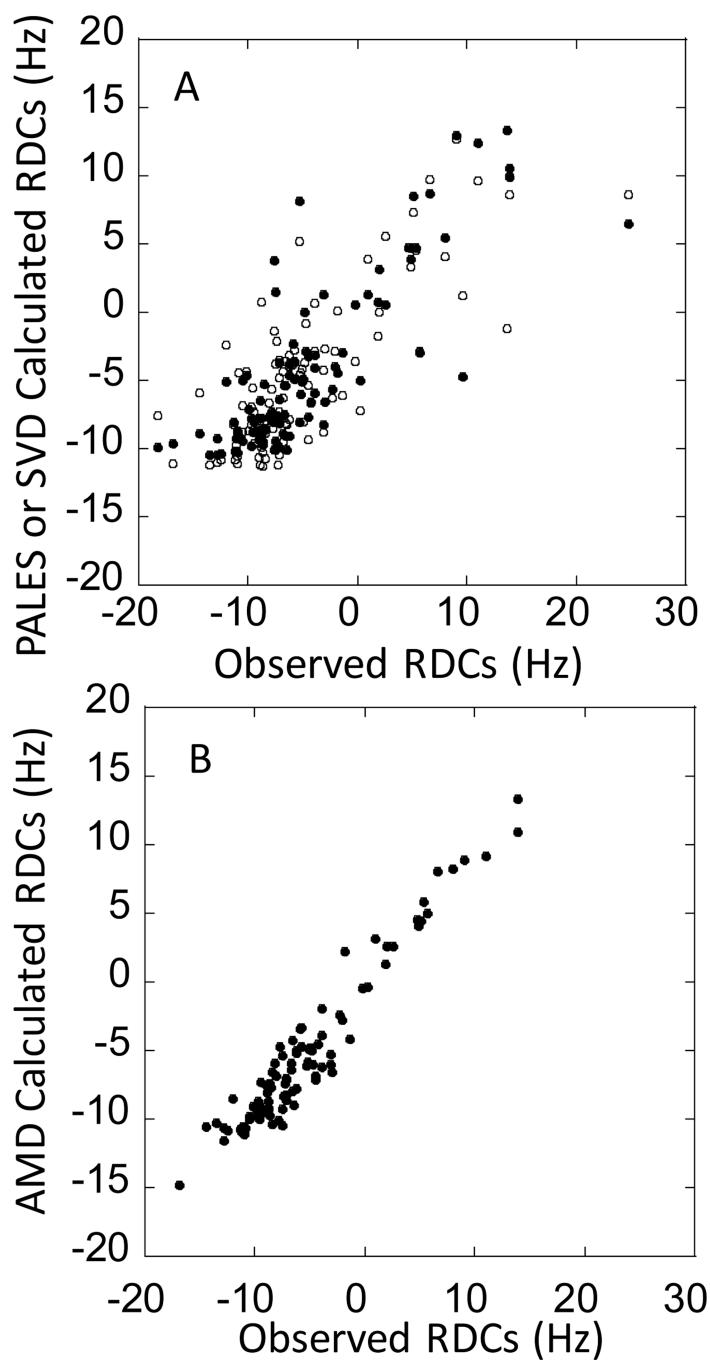
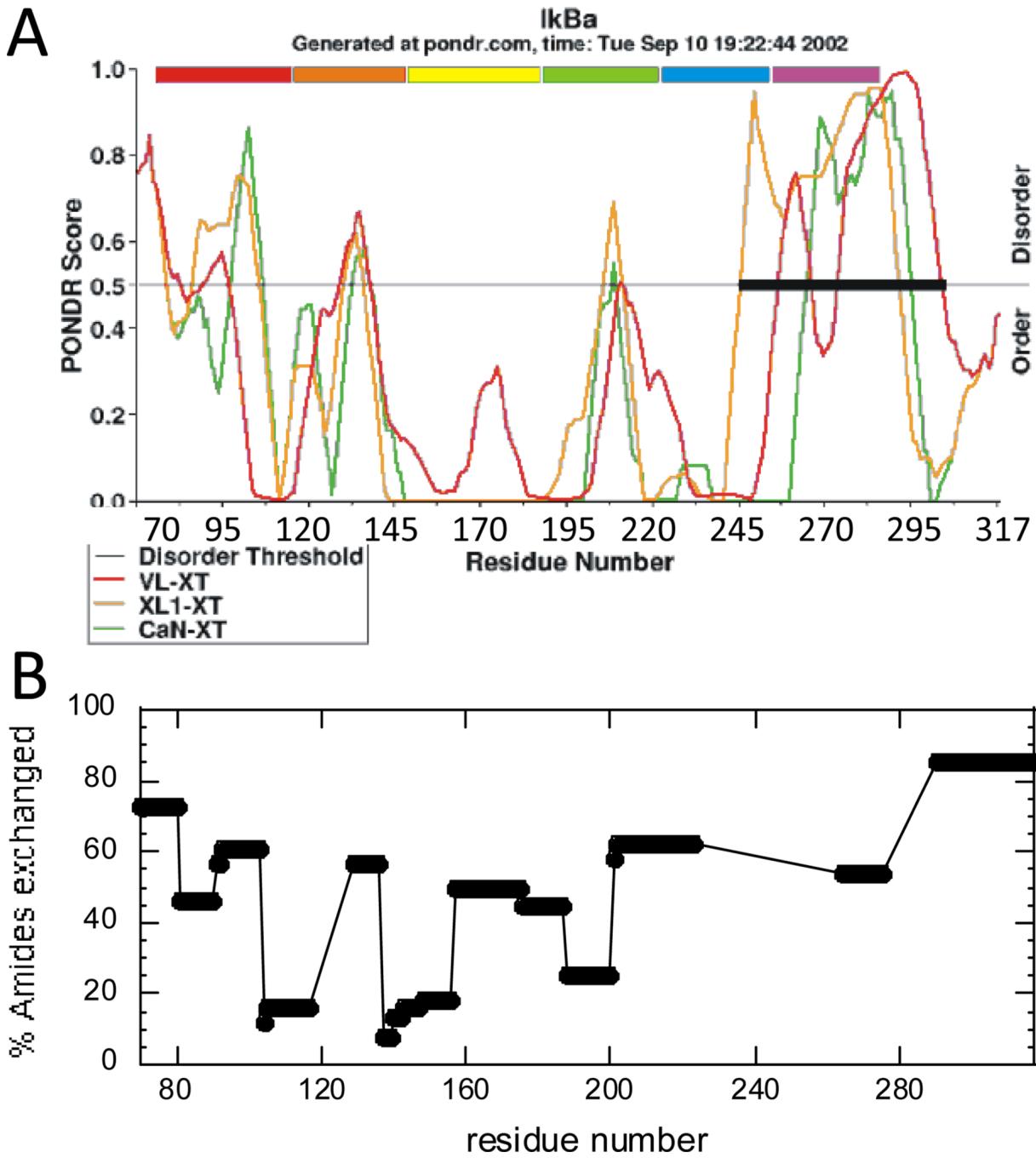


Figure 2.

(A) Observed vs. theoretical residual dipolar couplings measured by the program PALES for I κ B α (67–206) (closed symbols) and SVD (open symbols) using the crystal structure of the I κ B α •NF- κ B complex (PDB accession code 1IKN,¹⁰). (B) Observed vs. AMD-calculated residual dipolar couplings for I κ B α (67–206). The RDCs were measured as previously described²¹.

**Figure 3.**

(A) PONDR²³ analysis of the intrinsic disorder in the ankyrin repeat domain of IκBa. (B) Summary of native state amide H/D exchange results on free IκBa. IκBa(67–317) was allowed to exchange for 2 min, and after quenching, the protein was digested with pepsin and the amount of exchange in each peptide was analyzed by MALDI mass spectrometry²⁴.

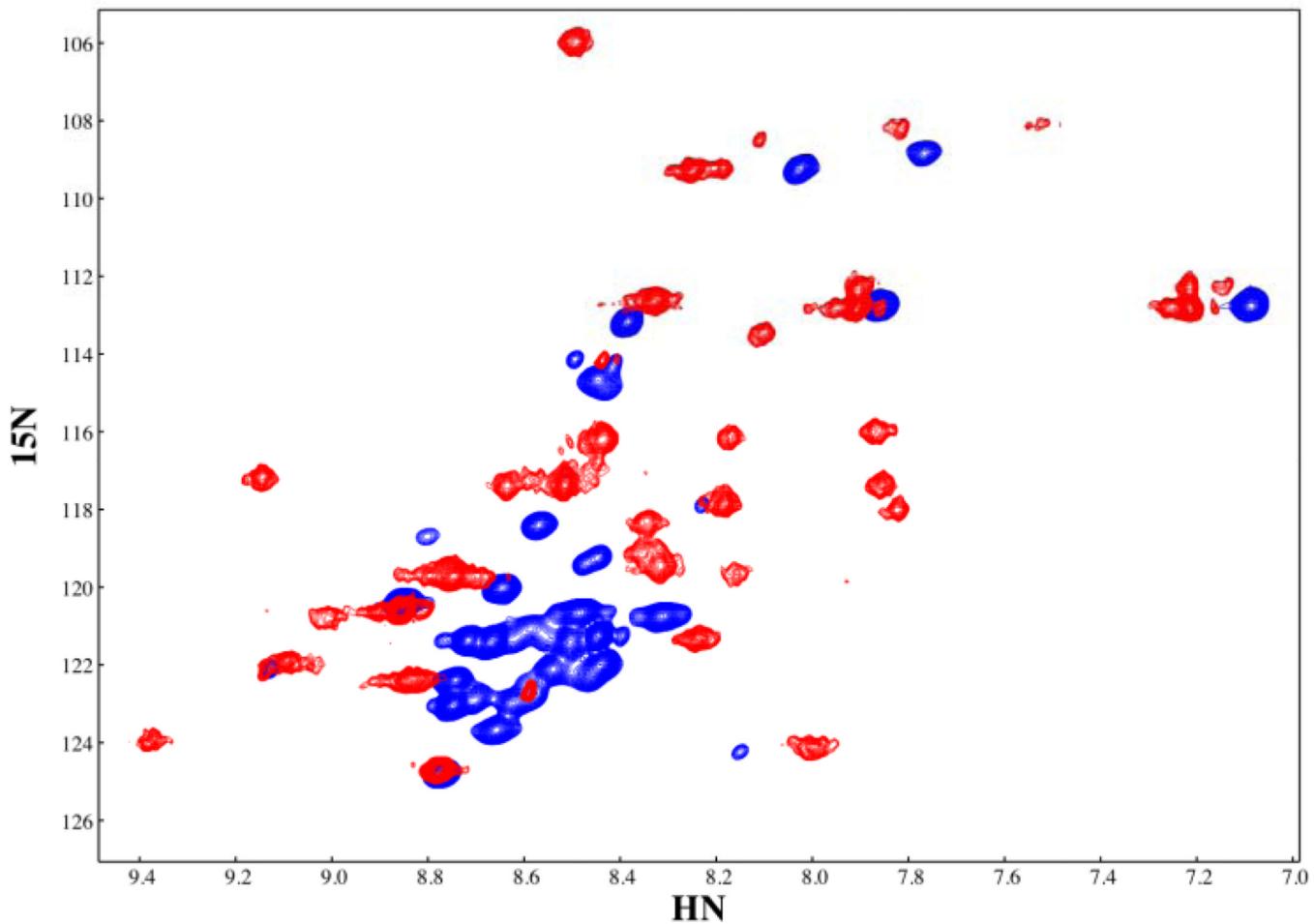
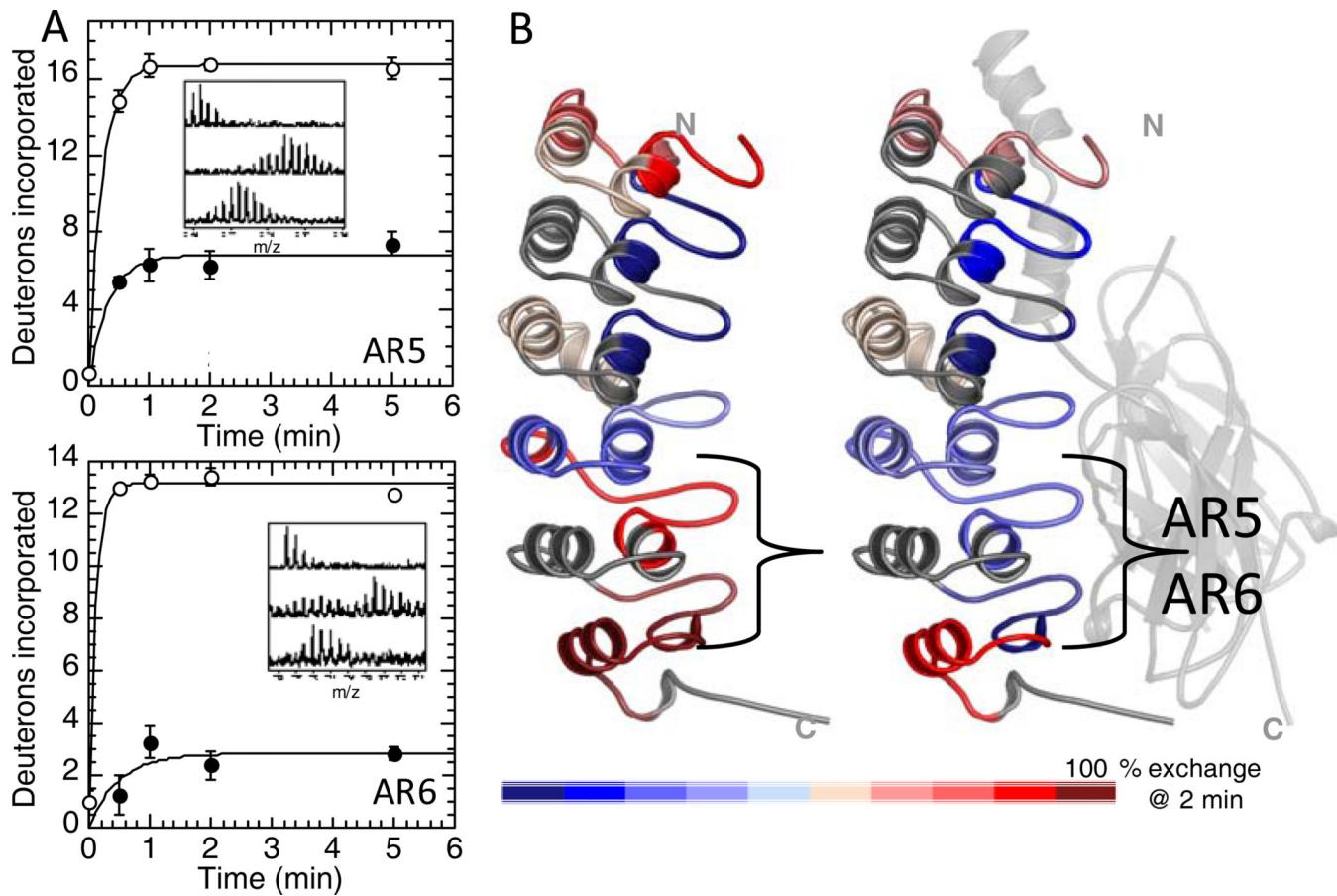
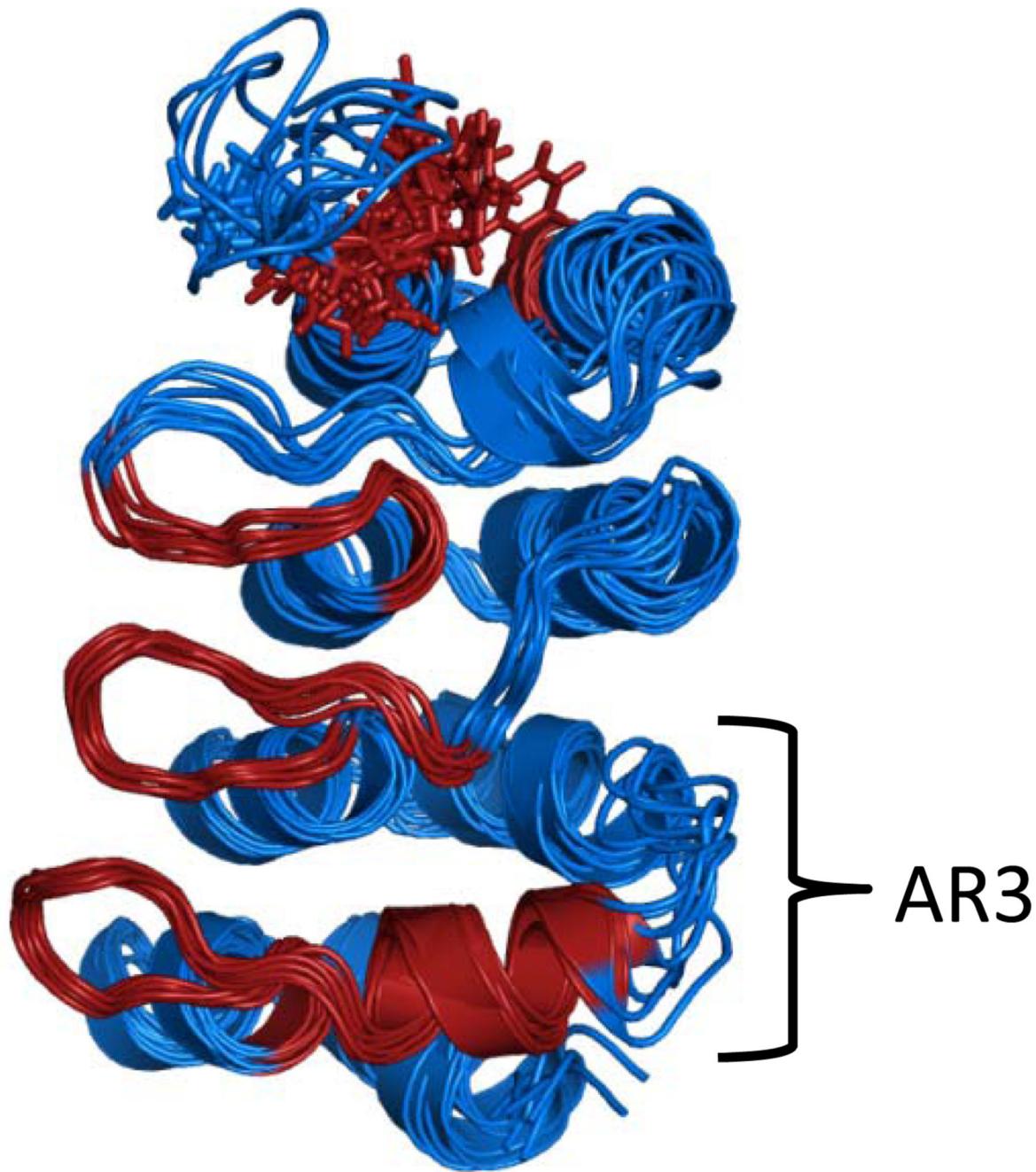


Figure 3.

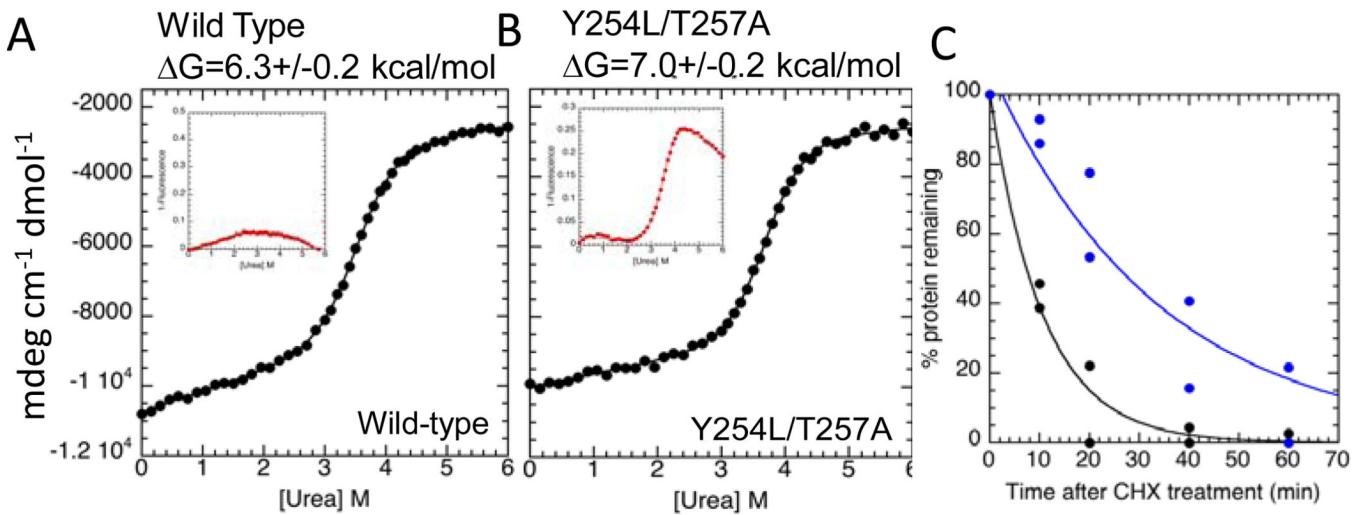
The HSQC spectra of free and IκBa-bound p65(289–321). The secondary chemical shifts of bound p65(289–321) show the characteristic positive values characteristic of helical regions, conforming to the helical areas seen in these same residues in the crystal structure of the IκBa•NF-κB complex (PDB accession code 1NFI,¹¹). The chemical shifts for free p65(289–321) are indicative of an unfolded peptide.

**Figure 5.**

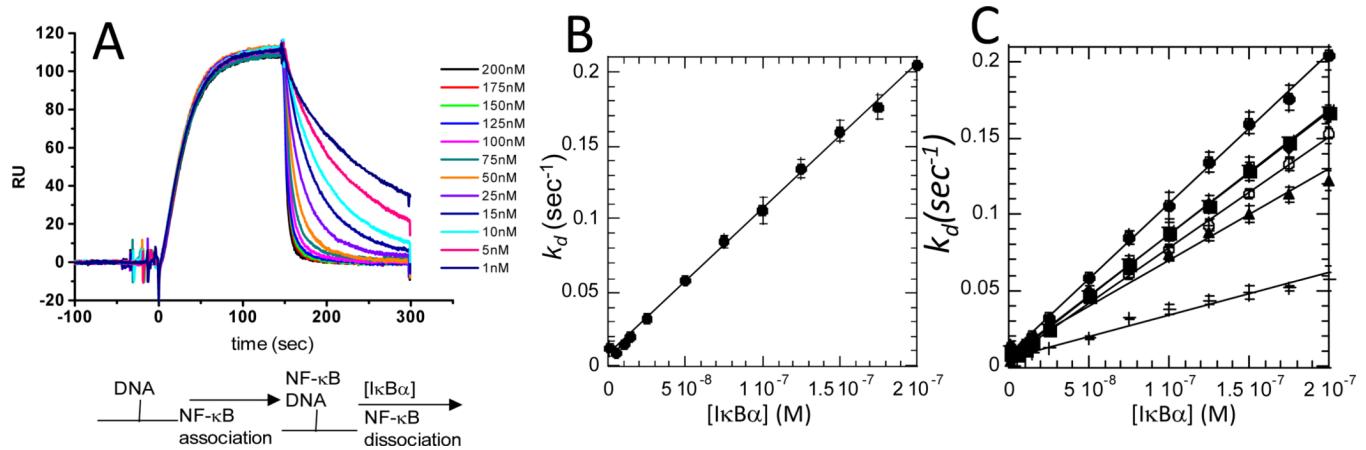
(A) Native state amide H/D exchange data for the region of I κ B α corresponding to the β -turn of AR5 (TOP) and AR6 (bottom) in the free-state (open circles) and in the NF- κ B-bound state (closed circles). (B) Structural summary of the amide H/D exchange data (red is highly exchanging and blue is slowly exchanging) for I κ B α in the free state (LEFT) and in the NF- κ B-bound state (RIGHT) showing that exchange is similar for most of the I κ B α molecule in each state, except for the β -turns of AR5 and AR6 that are exchanging much less in the bound state.

**Figure 6.**

Structural ensemble of I κ B α (67–206) showing representative structures from the AMD simulation. The molecule is colored red where NMR experiments indicated residues were undergoing conformational exchange (R_{ex})²¹.

**Figure 7.**

(A) Equilibrium unfolding experiments with wild type I κ B α and (B) Y254L,T257A mutant I κ B α . The insets show the change in fluorescence of W258, a naturally-occurring Trp258 in AR6. In the wild type protein, this residue does not change fluorescence appreciably with denaturant, however in the stabilized mutant, its fluorescence changes in a manner similar to the CD signal indicating it follows the major cooperative folding transition of the protein. (C) I κ B isoforms were measured by quantitative Western blot. The plot shows the levels of I κ B α (Y254L,T257A) (blue) and wild-type I κ B α (black) after cyclohexamamide treatment in NF- κ B $-/-$ cells.

**Figure 8.**

(A) Real-time SPR binding experiment in which κB -site DNA was bound to the streptavidin chip at $t=0$, then $NF-\kappa B(p50_{(19-363)}/p65_{1-35})$ was allowed to associate with the DNA, and finally varying concentrations of $I\kappa B\alpha$ were injected through the second sample loop and the dissociation rate constant (k_d) was measured⁴¹. A schematic of the binding events is shown below the graph. (B) Dissociation rate constants for active dissociation are plotted as a function of $I\kappa B\alpha$ variant concentration. (C) Comparison of the active dissociation rate constants of $I\kappa B\alpha$ folding variants: wild type $I\kappa B\alpha$ (●), $I\kappa B\alpha(C186P, A220P)$ (■), $I\kappa B\alpha(Q111G)$ (▲), $I\kappa B\alpha(Y254L/Q255H)$ (►), $I\kappa B\alpha(Y254L/T257A)$ (◀)⁴¹.