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Single-Molecule FRET Reveals the Native-State Dynamics of the IκBα Ankyrin Repeat Domain

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Abstract

Previous single-molecule fluorescence resonance energy transfer (smFRET) studies in which the second and sixth ankyrin repeats (ARs) of IκBα were labeled with FRET pairs showed slow fluctuations as if the IκBα AR domain was unfolding in its native state. To systematically probe where these slow dynamic fluctuations occur, we now present data from smFRET studies wherein FRET labels were placed at ARs 1 and 4 (mutant named AR 1–4), at ARs 2 and 5 (AR 2–5), and at ARs 3 and 6 (AR 3–6). The results presented here reveal that AR 6 most readily detaches/ unfolds from the AR domain, undergoing substantial fluctuations at room temperature. AR 6 has fewer stabilizing consensus residues than the other IκBα ARs, probably contributing to the ease with which AR 6 “loses grip”. AR 5 shows almost no fluctuations at room temperature, but a significant fraction of molecules shows fluctuations at 37 °C. Introduction of stabilizing mutations that are known to fold AR 6 dampen the fluctuations of AR 5, indicating that the AR 5 fluctuations are likely due to weakened inter-repeat stabilization from AR 6. AR 1 also fluctuates somewhat at room temperature, suggesting that fluctuations are a general behavior of ARs at ends of AR domains. Remarkably, AR 1 still fluctuates in the bound state, but mainly between 0.6 and 0.9 FRET efficiency, whereas in the free IκBα, the fluctuations extend to <0.5 FRET efficiency. Overall, our results provide a more complete picture of the energy landscape of the native state dynamics of an AR domain.

Keywords

single-molecule FRET; ankyrin repeat; intrinsically disordered protein

Introduction

The ankyrin repeat (AR) domain is one of the most common protein structures that functions primarily in protein–protein binding interactions.^{1,2} The SMART nonredundant database[‡]

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[‡]<http://smart.embl-heidelberg.de/>

reports 146,742 ARs in 25,863 proteins.^{3,4} AR-containing proteins are known to control transcriptional regulation, cell signaling, development, differentiation, apoptosis, and inflammatory responses, among other functions.² ARs are ~33-residue sequences featuring β -turn/ α -helix/ α -helix structures arranged linearly in repeating units.^{1,2} At least three ARs are required to make a stably folded AR domain.⁵ Sequence alignments of ARs reveal a consensus sequence, and proteins designed with all consensus ARs are much more stable than typical AR-containing proteins found in nature, which usually encompass only about 50% of the consensus residues.^{6,7} Interactions between ARs are more important for the AR domain stability than are interactions within one AR.^{1,2,5} We recently showed that substitution of any non-consensus residue with the corresponding consensus residue within I κ B α imparts additional stability, implying that each consensus residue contributes to the overall stability of the AR domain.⁸

I κ B α binds and inhibits NF κ B, an important transcription factor that regulates genes for cell growth, proliferation, apoptosis, and stress responses.⁹ I κ B α exerts tight control over the activity of NF κ B,¹⁰ and dysregulation of the NF κ B signaling system has been reported in heart failure, Alzheimer's disease, diabetes (types 1 and 2), and cancer, among other pathological conditions.¹¹ I κ B α has six ARs (Fig. 1). In the absence of NF κ B, ARs 5 and 6 of free I κ B α have properties of an intrinsically disordered protein, while the rest of the protein (ARs 1 through 4) remains well folded. ARs 5 and 6 in free I κ B α rapidly undergo amide hydrogen/deuterium (H/D) exchange, but amide exchange is markedly decreased when I κ B α is bound to NF κ B, providing strong evidence that the AR 5–6 region folds on binding.¹³ Intrinsic disorder is critically important for the ability of I κ B α to promote the dissociation of NF κ B from DNA transcription sites,¹⁴ as well as for establishing the rapid degradation rate of free I κ B α and maintaining low intracellular I κ B α levels.¹⁵ Intrinsic disorder in ARs 5 and 6 hampers the structural characterization of these regions by NMR or X-ray crystallography.

Single-molecule methods, for example, single-molecule fluorescence resonance energy transfer (smFRET),^{16,17} have been used to study conformational heterogeneity and dynamics of proteins.^{18–25} Recently, we presented smFRET data demonstrating that although the major conformation of free I κ B α resembles that of the NF κ B-bound one,^{26,27} free I κ B α undergoes heterogeneous fluctuations to a more extended structure on the millisecond time scale.¹² Our previous report used an I κ B α construct labeled at ARs 2 and 6, which we interpreted as mainly reporting on the fluctuations in the intrinsically disordered AR 6.

Here, we systematically probe the ARs beyond ARs 2 and 6 by placing FRET labels at ARs 1 and 4 (mutant named AR 1–4), at ARs 2 and 5 (AR 2–5), and at ARs 3 and 6 (AR 3–6) (Fig. 1a–d). Our smFRET results reveal that ARs 1 and 6 undergo fluctuations at room temperature, suggesting that detachment/unfolding of the end repeats is a general property of AR domains. AR 5, which H/D exchange analysis identified as disordered, shows almost no fluctuations at room temperature but fluctuates at 37 °C. Furthermore, stabilizing mutations that fold AR 6 attenuate the fluctuations of AR 5, which suggests the importance of inter-AR contacts for stabilizing ARs.

Results

AR 1–4 I κ B α shows fluctuations

NMR and H/D exchange experiments have demonstrated that ARs 1 through 4 are well folded, but that nearly all of the amides in AR 1 exchanged readily.^{13,28,29} To examine the single-molecule behavior of the N-terminal part of the I κ B α AR domain, we labeled I κ B α sites at positions 98 for AR 1 and 205 for AR 4 (denoted AR 1–4 I κ B α , Fig. 1a). AR 1–4

I κ B α and all other I κ B α mutants discussed here retained NF κ B binding ability (Fig. S1). Protein immobilization of free (not NF κ B-bound) AR 1–4 I κ B α and all other I κ B α mutants for total internal reflection fluorescence smFRET followed the scheme illustrated in Fig. 1e. Single-molecule trace analysis for generating histograms used the first ~200 traces that showed bona fide single molecules (i.e., clear donor–acceptor anti-correlation and single photobleaching events for both donor and acceptor). All traces showing bona fide single molecules were included in the analyses. At 25 °C, free AR 1–4 I κ B α revealed a major high FRET peak in the histogram (Fig. 2a). A typical FRET trace showing stable high FRET at 25 °C is shown in Fig. 2b. The observed FRET efficiency of 0.87 roughly reflected the distance between labeling sites in AR 1 and AR 4. Interestingly, the majority of individual trajectories showed stable high FRET (78%), but about 20% of the molecules showed fluctuations (Table 1). The fluctuations were within the high FRET regime (Fig. 2c) and so did not show up as a separate population in the FRET histogram.

Raising the temperature from 25 °C to 37 °C caused the number of fluctuating AR 1–4 I κ B α molecules to increase to 32% (Table 1), and the FRET histogram showed a small increase in low–mid FRET populations (Fig. 2d). An example of a FRET trace from an AR 1–4 I κ B α molecule showing low–mid FRET fluctuations at 37 °C is shown in Fig. 2e. Although the fraction of fluctuating AR 1–4 I κ B α molecules was lower than previously observed for the AR 2–6 I κ B α ,¹² AR 1–4 I κ B α molecules also explore FRET efficiencies in the high, mid, and very briefly in the low regime. At 37 °C, most of the AR 1–4 I κ B α molecules (66%) still showed stable high FRET for the duration of the data collection (typically 10–50 s until photobleaching of the acceptor dye occurred) (Table 1).

In our previous study, binding of NF κ B to the AR2-6 I κ B α completely dampened fluctuations. Therefore, it was surprising that NF κ B-bound AR 1–4 I κ B α molecules still showed fluctuating FRET signals. The fluctuations were initially observed in experiments in which the NF κ B–I κ B α complex was immobilized via a His₆ tag on I κ B α . We wondered whether the dissociation between NF κ B and I κ B α was fast enough that we were actually observing a large fraction of free I κ B α molecules. To exclude this possibility, we prepared NF κ B that was biotinylated at the N-terminus for immobilization on the neutravidin-coated slides and repeated the experiments on the NF κ B–I κ B α complexes. Figure 1f illustrates the immobilization scheme used for NF κ B-bound AR 1–4 I κ B α and all other NF κ B-bound I κ B α mutants. Even when this strategy allowed us to observe only NF κ B-bound I κ B α molecules, a significant fraction of the molecules appeared to be fluctuating (Table 1). The FRET histogram for NF κ B-bound AR 1–4 I κ B α at 25 °C (Fig. 2f) looked similar to the FRET histogram for free AR 1–4 I κ B α shown in Fig. 2a. NF κ B-bound AR 1–4 I κ B α fluctuated mostly within the high FRET regime, as shown in Fig. 2g. However, a small number of the fluctuating molecules briefly visited mid and low FRET. Similar percentages of NF κ B-bound AR 1–4 I κ B α molecules fluctuated at both 25 and 37 °C.

AR 3–6 I κ B α shows fluctuations similar to AR 2–6 I κ B α

To confirm the AR 6 dynamics observed with AR 2–6 previously¹² and to unveil potentially new behaviors, we constructed an I κ B α variant labeled at position 166 (AR 3) and at position 262 (the same AR 6 labeling site used previously for AR 2–6 I κ B α) (Fig. 1). The distance between the labeling sites on AR 3 and AR 6 was nearly the same as that of AR 2–6 I κ B α used previously (38.5 Å for AR 3–6 and 40.5 Å for AR 2–6, Fig. 1).¹² For free AR 3–6 I κ B α at 25 °C, the major peak of the FRET histogram was at 0.74 FRET efficiency (Fig. 3a), similar to that previously observed for AR 2–6 I κ B α .¹² Analysis of the individual FRET traces revealed a significant number of AR 3–6 I κ B α molecules showing fluctuations (44%, Table 1). Some of the fluctuations extended into the low FRET regime and resulted in an apparent low FRET population in the FRET histogram (Fig. 3a). The FRET trace from an example of one such fluctuating molecule is shown in Fig. 3b. Raising the temperature to 37

°C broadened the FRET histogram to the low–mid FRET regimes (Fig. 3c) and increased the fraction of fluctuating molecules to 78% of the total population (Fig. 3d; Table 1). As was the case for AR 2–6 IκBα, the secondary population at mid FRET is not the result of an independent population of molecules different from the main histogram population (at 0.74 FRET efficiency), but rather mostly the result of fluctuating molecules contributing data points to the mid and low FRET regimes of the FRET histogram.

Binding of AR 3–6 IκBα to NFκB markedly suppressed the fluctuations, and the FRET histogram showed a single high FRET peak (Fig. 3e). Examination of the individual FRET traces for NFκB-bound AR 3–6 IκBα revealed that the vast majority of the molecules (94% at 25 °C and 89% at 37 °C, Table 1) had stable high FRET, as exemplified in Fig. 3f, which recapitulates the observations made for NFκB-bound AR 2–6 IκBα.¹²

Comparison of AR 2–5 IκBα with AR 2–6 IκBα reveals that AR 5 fluctuates only at 37 °C whereas AR 6 fluctuates at both 25 °C and 37 °C

Both AR 5 and AR 6 were thought to be intrinsically disordered in free IκBα and to fold upon binding to NFκB, based on H/D exchange and NMR data.^{13,28,29} In order to probe AR 5 dynamics by smFRET, we constructed an IκBα variant labeled at position 128 (the same AR2 labeling site used previously for the studies on AR 2–6 IκBα)¹² and at position 234 (AR 5) (Fig. 1). Comparison of the single-molecule behaviors of the AR 2–5 and AR 2–6 IκBαs thus reports solely on the differences between the behavior of AR 5 and AR 6.

Previously, we showed that the FRET histogram for free AR 2–6 IκBα at 25 °C had two peaks, with the majority of molecules having stable high FRET but also a significant number of fluctuating molecules that contributed to an apparent low–mid FRET population in the FRET histogram (Fig. 4a and b).¹² A significant percentage (43%) of AR 2–6 IκBα molecules fluctuated at 25 °C (Table 1). In contrast, the FRET histogram for free AR 2–5 IκBα did not have an apparent low FRET population, and analysis of ~200 single-molecule traces revealed that the majority of the molecules (84%) had stable high FRET (Fig. 4c and d; Table 1). Only a few AR 2–5 IκBα molecules (14%) showed FRET fluctuation at 25 °C (Table 1). Consistent with the shorter inter-dye distance between the AR 2–5 labeling sites, AR 2–5 IκBα showed higher FRET efficiencies (~0.83) than AR 2–6 (~0.77). Increasing the temperature to 37 °C significantly increased the number of AR 2–5 IκBα molecules that were observed to fluctuate (Fig. 4e; Table 1). The FRET histogram reflected this large increase, revealing a concomitant low–mid FRET population similar to that seen for AR 2–6 IκBα at 25 °C (Fig. 4a). The apparent population at low–mid FRET was due to the presence of a small percentage of stable mid FRET molecules (11%) and a larger percentage of fluctuating molecules (37%). The single-molecule trace shown in Fig. 4f is a typical example of a fluctuating AR 2–5 IκBα molecule at 37 °C.

Binding of AR 2–5 IκBα to NFκB suppressed the fluctuations both at 25 °C and 37 °C. At 25 °C, the fraction of molecules showing stable high FRET increased from 86% to 95%, and at 37 °C, the fraction of molecules showing stable high FRET increased from 52% to 84% upon NFκB binding (Table 1). Consistent with these results, the FRET histogram for NFκB-bound AR 2–5 IκBα had only a major FRET peak with a slightly higher FRET efficiency (0.86) than free AR 2–5 IκBα (0.83) (Fig. 4g). Thus, the smFRET data are consistent with previous assertions that AR 5 becomes more stably folded upon binding to NFκB.¹³

Cross-correlation analysis compares the characteristic time scales of the fluctuations

In order to compare the characteristic time scale of the fluctuations between different ARs, we calculated the cross-correlation between the donor and acceptor fluorescence traces for AR 1–4, AR 2–5, and AR 3–6 IκBα at 37 °C (Fig. 5). Each graph was fitted well with bi-

exponential decay. Comparing the fitting parameters reveals that AR 1–4 I κ B α fluctuates with longer time scale than the others (Table 2). Both the fast and slow components of the decay are slower for AR 1–4 than those for AR 2–5 or AR 3–6 I κ B α by a factor of 1.6–2.8. The correlation amplitude of AR 1–4 I κ B α is significantly smaller than the others, consistent with its smaller low–mid FRET population.

AR 2–5 and AR 3–6 I κ B α fluctuate with similar time constants but with distinct population distribution. The fractional population of the faster component is only 39% for AR 2–5 while it is 62% for AR 3–6 I κ B α (Fig. 5b). As also demonstrated in the example traces (Figs. 3d and 4f), AR 3–6 fluctuates more frequently than AR 2–5 I κ B α . This can be understood if we consider that AR 3–6 I κ B α involves two unstable ARs (AR 5 and 6) while AR 2–5 I κ B α involves only one (AR 5).

Restoring the ankyrin consensus sequence in AR 6 stabilizes AR 5

AR 6 deviates so significantly from the consensus sequence for stable ARs that early sequence analyses did not recognize its sequence as an AR. AR 6 was determined to be an AR only after the crystal structure of I κ B α in complex with NF κ B was solved.^{26,27} Re-establishment of the TPLHLA consensus sequence in AR 6 by means of the Y254L/T257A (YLTA) mutations significantly stabilizes the protein³⁰ and “pre-folds” AR 6 according to amide H/D exchange and fluorescence experiments.³¹ Analysis of the single-molecule behavior of the YLTA-stabilized AR 2–6 I κ B α (denoted AR 2–6 YLTA) also revealed a near-complete suppression of the fluctuations.¹² To investigate YLTA-mediated stabilization beyond AR 6, we constructed an AR 2–5 I κ B α with the YLTA mutations in AR 6 (named AR 2–5 YLTA). AR 5 also deviates slightly from the ankyrin consensus sequence, and a second AR 2–5 variant with a mutation that restored the consensus sequence in AR 5 (A220P mutation; construct named AR 2–5 AP) was prepared to investigate stabilization by consensus mutations within AR 5.

The FRET histograms for the AR 2–5 YLTA and AR 2–5 AP mutant I κ B α s showed only a single high FRET peak at 25 °C (Fig. 6a and b), as was also seen for the wild-type (WT) AR 2–5 I κ B α . However, analysis of the individual traces revealed some differences. Although only 14% of the WT AR 2–5 I κ B α molecules showed fluctuations at 25 °C, introduction of the YLTA mutations markedly decreased this fraction to 5%. Notably, the AP mutation had little effect on the number of fluctuating molecules as compared to WT (Table 3). At 37 °C, the AP mutant showed a similar low FRET population in the FRET histogram (Fig. 6c) to what was observed for WT AR 2–5 I κ B α at the same temperature (Fig. 4e). In addition, the fractions of stable high FRET and fluctuating molecules were about the same for both WT AR 2–5 and AR 2–5 AP (Table 2). The low FRET population was again much less for AR 2–5 YLTA at 37 °C (Fig. 6d), as compared to WT AR 2–5 and AR 2–5 AP at the same temperature. A significant increase in the percentage of stable high FRET molecules was also observed for the YLTA variant (Table 2). Comparing histograms of WT AR 2–5 (Fig. 4e) and AR 2–5 AP and YLTA (Figs. 6c and d) at 37 °C suggested a larger fraction of mid FRET molecules for WT AR 2–5 than for AR 2–5 AP and YLTA. Indeed, WT AR 2–5 showed a larger fraction of stable mid FRET molecules at 37 °C (11%), whereas the fractions of AR 2–5 AP and YLTA molecules showing stable mid FRET at the same temperature were 2% and 5%, respectively. These small percentages result from few molecules that appear to be in a long-lived stable mid FRET state. Such slight differences in the number of these molecules causes slight differences in the low–mid FRET populations in the histograms. Overall, restoration of the AR consensus sequence in AR 6 decreased the fluctuations of AR 5, whereas restoration of the AR consensus sequence in AR 5 did not change the behavior of AR 5 significantly.

Discussion

AR 1 fluctuates in both free and NF κ B-bound I κ B α

The smFRET results showed that a significant number of free AR 1–4 I κ B α molecules fluctuated even at 25 °C. This was a surprise because AR 1–4 I κ B α gave well-resolved NMR cross-peaks and had high-order parameters throughout all four ARs.²⁸ On the other hand, most of the amides in AR 1 showed rapid H/D exchange.¹³ The FRET fluctuations observed for AR 1–4 I κ B α could have been due to fluctuations of AR 1 or AR 4, or both. However, based on H/D exchange data on the full-length protein, it is more likely that the fluctuations are due to AR 1. AR 1 can only form stable contacts with one other AR (AR 2), and it is likely that insufficient inter-AR contacts result in a higher propensity for AR 1 to loosen or “lose its grip”. AR 6 also fluctuates in the free state,¹² but such fluctuations are driven in part by the substantial deviation of AR 6 from the consensus sequence for stable ARs. AR 1 contains more consensus residues than AR 6; thus, the results presented here suggest that the fluctuations are due more to the fact that the AR is at the end of the I κ B α AR domain and not only a result of consensus sequence deviations in AR 6. Our cross-correlation results suggest that such fluctuation of AR 1 out of the cooperatively folded ARs 1–4 happens more rarely and slowly compared to the sequence-inherent fluctuation at the other end of I κ B α .

Remarkably, the AR 1–4 fluctuations were still present in the NF κ B-bound state. The nuclear localization sequence of NF κ B lies over the top of AR 1 in I κ B α ,²⁶ and this interaction provides nearly 8 kcal/mol of binding affinity in the complex.³² On the other hand, the crystallographic *B*-factors for this part of the NF κ B–I κ B α complex are very high, implying disorder in this region.²⁶ Comparing the smFRET results to previous H/D exchange results is informative. Whereas nearly all amides in AR 5 and AR 6 undergo rapid H/D exchange in the free state, more than 10 amides become slowly exchanging in the NF κ B-bound state, consistent with the well-folded nature of the bound AR 5–6 region¹³ and also with the near-complete dampening of fluctuations upon binding.¹² In contrast, the H/D exchange in AR 1 only slightly decreases (1–2 amides fewer) upon binding to NF κ B.¹³ It is possible that this part of the NF κ B–I κ B α binding interaction is a new example of a “fuzzy” complex in which tight binding affinity is achieved with structures that remain disordered.³³

The observation that AR 1 still fluctuates in the NF κ B-bound state is very interesting. NF κ B signaling is activated in response to receptor–ligand binding events that cause activation of the I κ B kinase, IKK. IKK then phosphorylates I κ B α , targeting it for ubiquitination and subsequent degradation. The phosphorylation at residues 32 and 36³⁴ and ubiquitination at residues 21 and 22^{35–37} in the disordered N-terminus of I κ B α do not significantly alter the binding affinity between NF κ B and I κ B α ,³⁸ and it is therefore interesting to speculate about how the NF κ B–I κ B α complex “comes apart” after phosphorylation/ubiquitination to release NF κ B for its nuclear translocation. The affinity of I κ B α for NF κ B is in the picomolar range,³² and in the absence of IKK, the intracellular half-life of the complex is many hours.^{14,39} Given the scenario of a very tightly bound complex for which the affinity is unchanged upon phosphorylation and ubiquitination, we previously speculated that the proteasome actually proteolyzes the I κ B α beginning at the N-terminus of I κ B α while it is still bound to NF κ B in order to release active NF κ B.³⁹ This scenario would not be possible if the “hot spot” were so tightly bound that the proteasome could not digest past the NF κ B-bound AR 1. The fact that AR 1 still fluctuates in the NF κ B-bound form would allow the proteasome an opportunity to proteolyze past the hot spot, releasing bound NF κ B.

The ARs at both ends of the AR domain fluctuate

In our previous study of AR 2–6 I κ B α , we indicated that the observed fluctuations were so heterogeneous as to preclude assignment of states to defined structures. Instead, we imagined a broad molecular ensemble or molten globular state. At about the same time, Choi et al. observed a similar behavior in intrinsically disordered proteins and suggested that slow fluctuations may be characteristic of intrinsically disordered globules as opposed to stable low FRET, which they observed in an extended coil.²¹ The fact that AR 1 was also observed to fluctuate indicates that the ARs on either end of the domain can become “unglued” from the folded I κ B α AR domain. In retrospect, this result makes sense because several protein folding studies have suggested that stabilization of AR domains is largely due to inter-domain contacts, which would necessarily be less for the ARs on the ends. We originally suggested that AR 6 might be prone to disengage/unfold due to the dearth of consensus residues in this AR. The fact that AR 1 fluctuates less than AR 6 at 25 °C suggests that the presence of consensus residues is important, but even ARs that contain a sufficient number of consensus residues will fluctuate if they are on the end of the AR domain.

AR 6 frays more readily than AR 5

For both AR 2–6 and AR 3–6 I κ B α s, we observed large numbers of fluctuating molecules at 25 °C, with a concomitant increase in the number of fluctuating molecules at 37 °C. The results from AR 3–6 I κ B α confirm our previous observations that AR 6 fluctuates even at 25 °C and more at 37 °C. On the other hand, almost none of the AR 2–5 I κ B α molecules fluctuated at 25 °C. This was a surprising observation because previous amide H/D exchange experiments showed that both ARs exchanged all their amides within a few minutes, hinting that they were equally disordered.¹³ In contrast, the smFRET studies showed that AR 5 was markedly less dynamic at 25 °C than AR 6. Fluctuations of AR 5 emerged only at higher temperatures (37 °C). Comparing the cross-correlation results between AR 2–5 and AR 3–6 quantitatively confirms that AR 6 fluctuates more frequently than AR 5, reflecting their difference in positioning. The propensity of AR 6 to become “unglued” from the rest of the AR domain is most likely due to a combination of its being on the end of the AR domain and the dearth of consensus residues in AR 6.

To investigate whether deviation from the TPLHLA consensus sequence for ARs contributes to increased fraying of AR 6 in I κ B α , we used a “pre-folded” mutant with the YLTA mutations we had studied previously.^{30,31} Restoration of the consensus sequence in AR 6 by introduction of the YLTA mutations suppresses fluctuations in this AR.¹² In addition, the fluctuations observed in AR 2–5 I κ B α at 37 °C were markedly dampened by the YLTA mutations. Thus, pre-folding of AR 6 may facilitate better inter-AR contacts with AR 5. Interestingly, restoration of the AR consensus sequence in AR 5 by way of the A220P (AP) mutation did not significantly alter the fluctuating fraction of AR 2–5 molecules.

Our results allow for a deeper understanding of the role of individual residues within the TPLHLA consensus sequence motif, which has been suggested to facilitate effective inter- and intra-AR contacts.^{7,8,40,41} Restoration of the proline in AR 5 stabilizes I κ B α to urea denaturation³⁰ but does not affect the H/D exchange, ubiquitin-independent proteasomal degradation,³¹ or the propensity of AR 5 to fluctuate at 37 °C (vide infra). In contrast, restoration of the leucine and alanine residues in AR 6 of I κ B α both stabilizes the protein somewhat to urea denaturation and results in a more well-folded AR 6, leading to decreased H/D exchange, slower proteasomal degradation,³¹ and reduction of AR 6 fluctuations.¹² Although the term “stabilization” can mean many different things, the YLTA consensus residues cause stabilization in every sense of the word, increasing the resistance of the entire

protein to denaturant, dampening H/D exchange, and suppressing the slow fluctuations observed in the AR containing the mutations, AR 6, and the adjacent AR, AR 5.

Methods

I κ B α purification and labeling for FRET

All the WT cysteines in I κ B α were replaced with serines by site-directed mutagenesis before introduction of cysteines at positions 98 and 205 (for AR 1–4), 128 and 234 (for AR 2–5), 128 and 262 (for AR 2–6), and 166 and 262 (for AR 3–6). The AP and YLTA mutations were introduced to the AR 2–5 construct by site-directed mutagenesis.

Expression and purification of the I κ B α constructs followed the protocols described previously.^{12,42} Immediately before labeling, I κ B α was passed through a size-exclusion column in labeling buffer [25 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM TCEP] and concentrated to ~100 μ M. The detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) was added to a 5-mM final concentration after size exclusion and before the concentration step to reduce protein aggregation. Dimethyl sulfoxide (DMSO) was also added to 10% (v/v) to increase dye solubility during the reaction. An equimolar mixture of maleimide-conjugated Alexa 555 and Alexa 647 (Life Technologies) was prepared in 10-fold excess of I κ B α (1 mM for each fluorophore in dry DMSO) and added to the protein (250 μ L volume) in 2- μ L aliquots at a time, with 5 min of reaction time between each aliquot addition. The reaction mixture was protected from light and incubated at room temperature for 1 h, followed by 4 °C overnight. The labeled protein was isolated by gel filtration in a Sephadex G-25 resin (Sigma) using isolation buffer (25 mM Tris, pH 7.2, 150 mM NaCl, 1 mM EDTA, 10 mM Chaps, and 10% DMSO), followed by a final size-exclusion step into I κ B α buffer (25 mM Tris, pH 7.2, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT). Chaps was added for a 5-mM final concentration after the size-exclusion step to minimize protein aggregation during concentration and handling. Labeling efficiencies were determined as described previously.¹² Binding of the labeled proteins was confirmed by surface plasmon resonance (Supplementary Fig. S1).

smFRET measurements and analysis

We used total internal reflection fluorescence microscopy for imaging as described previously.^{12,43} Free I κ B α was immobilized following the scheme described previously.¹² NF κ B-bound I κ B α (except for AR 2–6) was immobilized through biotin-labeled NF κ B, where biotin was conjugated to the p65 monomer in the p50/p65 heterodimer. Purified murine p65 (190–321) in I κ B α buffer was singly biotinylated at Cys197 by incubation with biotin-conjugated PEO maleimide (Thermo Scientific Pierce Chemicals; 1:1 p65: biotin-maleimide molar ratio), nutation at room temperature for 1 h, and purification immediately by size-exclusion chromatography on a S75 Superdex 16/60 column in high salt buffer [500 mM NaCl, 10 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM sodium azide, and 0.005% (v/v) P20]. Murine p50 (248–350) was purified by size-exclusion chromatography as above. To form the p50/p65–biotin heterodimer, a 50-fold excess of unbiotinylated p50 was incubated with biotinylated p65 at room temperature for 1 h and subsequently at 4 °C overnight. For imaging, the biotin–NF κ B/I κ B α complex (pre-incubated in a 3:1 biotin–NF κ B:I κ B α ratio for at least 1 h at room temperature, or overnight at 4 °C) was added to the neutravidin-coated surface described previously,¹² but in these experiments, the surface lacked the biotinylated anti-His₅ antibody used for immobilizing free His₆-tagged I κ B α . Thus, the biotin–NF κ B/I κ B α complex was bound specifically to the neutravidin immobilized to the surface, which ensured that every I κ B α imaged was bound to NF κ B.

Cross-correlation analysis of single-molecule traces

In order to estimate the characteristic time scale of fluctuations, we analyzed the cross-correlation of the fluorescence time traces. The correlation function for one molecule is defined as $C(\tau) = \left\{ \int_0^{T-\tau} (D(t) - \bar{D}) \times (A(t+\tau) - \bar{A}) dt \right\} / \left\{ (T-\tau) \times (\bar{D} + \bar{A}) \right\}$, where $D(t)$ and $A(t)$ are donor/acceptor fluorescence signals, $\bar{D}$ and $\bar{A}$ are their means over the time range $(0, T-\tau)$, and T is the total length of the selected trace. We normalized the correlation function by the sum of donor and acceptor signals instead of both multiplied, because extremely low or high FRET molecules can give very large correlation amplitude due to the small amplitude of either signal. We selected only the traces longer than 10 s and calculated the correlation function up to 5 s. We excluded traces that exhibited severe fluctuation or gradual drift of the total fluorescence signal because they give strong positive correlation unrelated to the inherent dynamics of the molecules. The histograms resulting from the remaining traces after this second selection were indistinguishable from the histograms presented in Figs. 2–5. We fitted the correlation curves to bi-exponential decay to compare the characteristic time scale of the fluctuations.

Supplementary Material

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Abbreviations used

AR	ankyrin repeat
AP	A220P
YLTA	Y254L/T257A
WT	wild type
smFRET	single-molecule fluorescence resonance energy transfer
H/D	hydrogen/deuterium
EDTA	ethylenediaminetetraacetic acid
Chaps	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DMSO	dimethyl sulfoxide

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Fig. 1.

Crystal structure of IκBα (green) bound to NFκB (gray) (Protein Data Bank ID: 1NFI). All six cysteines in WT IκBα were replaced with serines, followed by introduction of cysteine pairs at positions (a) 98 and 205 (yellow spheres, for AR 1–4 IκBα), (b) 128 and 234 (blue spheres, for AR 2–5 IκBα), (c) 128 and 262 (purple spheres, for AR 2–6 IκBα), and (d) 166 and 262 (red spheres, for AR 3–6 IκBα). IκBα was labeled for FRET with a maleimide-conjugated Alexa 555 and Alexa 647 FRET pair. Previously reported smFRET results from AR 2–6¹² are reproduced here to aid direct comparison with the other constructs. Red lines between the colored residues depict the intramolecular distances between labeling sites. For the Alexa 555–Alexa 647 pair ($R_0 = 51$ Å), the expected FRET efficiencies are 0.96 for AR 1–4, 0.94 for AR 2–5, 0.80 for AR 2–6, and 0.84 for AR 3–6. (e) Schematic depicting how free IκBα was immobilized by way of an N-terminal His₆ tag fused to the protein, which bound to a biotinylated anti-His₅ antibody on the quartz slide. (f) Schematic depicting how NFκB-bound IκBα measurements were made; the quartz slide lacked the anti-His₅ antibody, and the NFκB–IκBα complex was immobilized by a biotin placed at the N-terminus of p65 in the p50/p65 NFκB heterodimer, which bound onto the neutravidin-coated surface.

Fig. 2.

smFRET histograms and sample traces of AR 1–4 I κ B α under various conditions. The major peak in each histogram is labeled with the FRET efficiency at the peak maximum. (a) The FRET histogram for free AR 1–4 I κ B α at 25 °C had a major high FRET peak centered at 0.87 FRET efficiency. (b) A sample AR 1–4 I κ B α single-molecule trace showing stable high FRET at 25 °C (green trace = donor Alexa 555; red trace = acceptor Alexa 647; blue trace = FRET efficiency). (c) Even though a second lower FRET peak was not observed in the FRET histogram for AR 1–4 I κ B α at 25 °C, examination of individual single-molecule traces revealed that a large fraction of the molecules showed fluctuations mainly within the high FRET regime. An example of a fluctuating FRET trace from one molecule of AR 1–4 I κ B α at 25 °C is shown. (d) Incubation of free AR 1–4 at 37 °C resulted in an increase in the number of fluctuating molecules. The FRET histogram revealed a major population similar to the free AR 1–4 I κ B α at 25 °C, centered at 0.84 FRET efficiency with a tail at lower FRET efficiencies. Examination of individual FRET traces revealed that the tail in the histogram was due to a large fraction of fluctuating AR 1–4 I κ B α molecules. (e) Sample trace of a fluctuating AR 1–4 I κ B α molecule at 37 °C. (f) Binding of AR 1–4 I κ B α to NF κ B at 25 °C did not completely suppress fluctuations, and the histogram showed a tail at low–mid FRET. (g) Sample trace of a fluctuating NF κ B-bound AR 1–4 I κ B α molecule at 25 °C.

Fig. 3. smFRET histograms of AR 3–6 IκBα under various conditions and their corresponding sample traces. The major peak in each histogram is labeled with the FRET efficiency at the peak maximum. (a) Free AR 3–6 IκBα at 25 °C had a major high FRET peak at 0.74 FRET efficiency. However, a pronounced number of molecules fluctuated, which gave rise to low–mid FRET populations. (b) Sample trace of a fluctuating AR 3–6 IκBα molecule at 25 °C. (c) When incubated at 37 °C, free AR 3–6 IκBα showed an increased number of fluctuating molecules that substantially populated the low–mid FRET regimes. (d) Sample trace of a fluctuating AR 3–6 IκBα molecule at 37 °C. (e) NFκB-bound AR 3–6 IκBα at 25 °C revealed a histogram with a single high FRET peak. (f) Sample trace of a stable high FRET, NFκB-bound AR 3–6 IκBα at 25 °C.

Fig. 4. smFRET histograms of AR 2–6 IκBα (a) and AR 2–5 IκBα (c, e, and g) under various conditions and their corresponding sample traces (b, d, f, and h). The major peak in each histogram is labeled with the FRET efficiency at the peak maximum. (a) At 25 °C, free AR 2–6 IκBα revealed not only a major stable high FRET population centered at 0.77 FRET efficiency but also a large number of fluctuating molecules (43%), as demonstrated by low–mid FRET populations in the histogram. (b) Sample trace of a fluctuating free AR 2–6 IκBα at 25 °C. (c) Free AR 2–5 IκBα at 25 °C demonstrated a major high FRET population and few fluctuating molecules. (d) Sample trace of a stable high FRET molecule for free AR 2–5 IκBα at 25 °C. (e) Higher temperature (37 °C) increased the number of fluctuating free AR 2–5 IκBα molecules, which resulted in increased low–mid FRET populations. (f) Sample trace of a free AR 2–5 IκBα molecule fluctuating at 37 °C. (g) FRET histogram of NFκB-bound AR 2–5 IκBα at 25 °C showing a single peak at high FRET. (h) Sample trace of an NFκB-bound AR 2–5 IκBα molecule (the traces at 25 °C and 37 °C all resembled this one).

Fig. 5.

Cross-correlation analysis of IκBα fluctuations. Cross-correlation curves for AR 1–4, AR 2–5, and AR 3–6 obtained by averaging the curves for 156, 231, and 93 selected molecules, respectively. Each curve was fitted with a bi-exponential decay, defined as $C(\tau) = A_{fast}e^{-\tau/\tau_{fast}} + A_{slow}e^{-\tau/\tau_{slow}}$.

Fig. 6.

smFRET histograms of AR 2–5 mutant I κ B α s. At 25 °C, both free AR 2–5 AP (a) and free AR 2–5 YLTA (b) showed 80% stable high FRET molecules. Both constructs behaved like WT AR 2–5 at 25 °C. (c) At 37 °C, the AP mutation failed to stabilize AR 2–5, and the resulting histogram resembled the one for WT AR 2–5 at the same temperature (Fig. 4e). (d) The YLTA mutations had an observable effect on the stability of AR 2–5 at 37 °C, which narrowed the histogram and increased the number of stable high FRET molecules.

Table 1

Percentage of molecule behaviors

	AR 2-6			AR 2-5			AR 1-4			AR 3-6						
	25 °C	37 °C	+NFKB 25 °C	25 °C	37 °C	+NFKB 25 °C	25 °C	37 °C	+NFKB 25 °C	25 °C	37 °C	+NFKB 25 °C				
Stable high FRET	52	38	78	82	84	52	95	84	78	66	81	78	56	21	94	89
Fluctuating	43 ^a	62 ^a	20 ^a	16 ^a	14	37	5	16	19	32	16	19	44	78	4	8

About 200 molecules were surveyed for each condition. Dominant molecule behaviors are in boldface. Conditions failing to add up to 100% of the molecules include a small number of molecules with unstable-mid FRET behavior, which is not reported in the table. Categorization of FRET behaviors: high FRET, 0.6–1.0 FRET efficiency; mid FRET, 0.3–0.6 FRET efficiency; low FRET, 0–0.3 FRET efficiency. We defined a fluctuation as the crossing of the observed FRET efficiency with >0.2 FRET efficiency units.

^aThe number of fluctuating molecules reported here includes those that are fluctuating within the range of high FRET (0.6–0.9 FRET efficiency), whereas the number reported in our previous paper¹² did not include molecules fluctuating within the high FRET regime.

Table 2

Amplitudes and time constants from the cross-correlation analysis

	AR 1-4	AR 2-5	AR 3-6
τ_{fast} (s)	0.27 ± 0.03	0.17 ± 0.02	0.16 ± 0.02
τ_{slow} (s)	3.6 ± 0.1	1.7 ± 0.04	1.3 ± 0.05
A_{fast}	44 ± 3	65 ± 6	115 ± 7
A_{slow}	62 ± 1	101 ± 2	71 ± 3

Table 3

Percentage of molecule behaviors for AR 2–5 variants

	<u>AR 2–5 wt^a</u>		<u>AR 2–5 AP</u>		<u>AR 2–5 YLTA</u>	
	25 °C	37 °C	25 °C	37 °C	25 °C	37 °C
Stable high FRET	84	52	80	49	93	71
Fluctuating	14	37	19	49	5	24

About 200 molecules were surveyed for each condition. Dominant molecule behaviors are in boldface. Conditions failing to add up to 100% of the molecules include a small number of molecules with stable–mid FRET behavior, which is not reported in the table.

^aReproduced from Table 1 to ease comparison with the AR 2–5 AP and YLTA variants.