

Article

Binding of NF κ B Appears to Twist the Ankyrin Repeat Domain of I κ B α Morten Beck Trelle,¹ Kristen M. Ramsey,¹ Taehyung C. Lee,² Weihua Zheng,³ Jorge Lamboy,¹ Peter G. Wolynes,³ Ashok Deniz,² and Elizabeth A. Komives^{1,*}¹Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California; ²Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, California; and ³Department of Chemistry, Rice University, Houston, Texas

ABSTRACT Total internal reflection fluorescence-based single-molecule Förster resonance energy transfer (FRET) measurements were previously carried out on the ankyrin repeat domain (ARD) of I κ B α , the temporally regulated inhibitor of canonical NF κ B signaling. Under native conditions, most of the I κ B α molecules showed stable, high FRET signals consistent with distances between the fluorophores estimated from the crystal structures of the NF κ B(RelA/p50)-I κ B α complex. Similar high FRET efficiencies were found when the I κ B α molecules were either free or in complex with NF κ B(RelA/p50), and were interpreted as being consistent with the crystallographically observed ARD structure. An exception to this was observed when the donor and acceptor fluorophores were attached in AR3 (residue 166) and AR6 (residue 262). Surprisingly, the FRET efficiency was lower for the bound I κ B α molecules (0.67) than for the free I κ B α molecules (0.74), apparently indicating that binding of NF κ B(RelA/p50) stretches the ARD of I κ B α . Here, we conducted confocal-based single-molecule FRET studies to investigate this phenomenon in greater detail. The results not only recapitulated the apparent stretching of the ARD but also showed that the effect was more pronounced when the N-terminal domains (NTDs) of both RelA and p50 were present, even though the interface between NF κ B(RelA/p50) and I κ B α encompasses only the dimerization domains. We also performed mass spectrometry-detected amide hydrogen/deuterium exchange (HDXMS) experiments on I κ B α as well as I κ B α bound to dimerization-domain-only constructs or full-length NF κ B(RelA/p50). Although we expected the stretched I κ B α to have regions with increased exchange, instead the HDXMS experiments showed decreases in exchange in AR3 and AR6 that were more pronounced when the NF κ B NTDs were present. Simulations of the interaction recapitulated the increased distance between residues 166 and 262, and also provide a plausible mechanism for a twisting of the I κ B α ARD induced by interactions of the I κ B α proline-glutamate-serine-threonine-rich sequence with positively charged residues in the RelA NTD.

INTRODUCTION

The NF κ B pathway is one of the central regulators of inflammatory responses in virtually every type of animal cell. Since its discovery almost 30 years ago (1), NF κ B has been implicated in regulation of cell growth/proliferation, apoptosis, and various stress responses, and is misregulated in numerous disease states (2). NF κ B exists in the resting cell as a panoply of homo- and heterodimers of the NF κ B family of proteins (RelA (p65), RelB, c-Rel, p50, and p52) bound to members of a family of inhibitor proteins (I κ Bs) (3). The best-characterized inhibitor is I κ B α , which binds preferentially to p50/RelA heterodimers with picomolar affinity (4). The full-length I κ B α is composed of three major regions: an N-terminal signal response region of ~70 amino acids, and ankyrin repeat domain (ARD) of ~220 amino acids, and a C-terminal proline-glutamate-serine-threonine-rich sequence (PEST) that extends from residues 275–317 (5).

NF κ B proteins have a common structural architecture that consists of a Rel-homology domain (RHD) and a transacti-

vation domain. The RHD is composed of an N-terminal domain (NTD) followed by a short linker to the dimerization domain, which is responsible for dimerization of NF κ B monomers and also serves as the binding domain for I κ Bs. The DNA-binding site is centered at the small linker connecting the NTD and dimerization domain, and DNA makes contacts with both the NTD and the dimerization domains when bound (6). Structural studies of the NF κ B(RelA/p50)-I κ B α interaction include x-ray crystal structures of the NF κ B(RelA/p50)-I κ B α complex, as determined by two independent groups (7,8). The I κ B α structure in the complex (shown schematically in Fig. 1 A) includes six stacked ankyrin repeats (ARs) and a C-terminal PEST. Each of these crystal structures captured only a portion of the entire complex, with the structure determined by Jacobs and Harrison (7) capturing (in one molecule of the two in the asymmetric unit) the interaction between the RelA nuclear localization signal (NLS) and the first ankyrin repeat of I κ B α , and the structure determined by Huxford et al. (8) capturing the interaction between the PEST and the RelA DNA-binding region.

Mass spectrometry (MS), NMR spectroscopy, and single-molecule Förster resonance energy transfer (smFRET) studies have all revealed flexible regions in free and NF κ B-bound

Submitted August 7, 2015, and accepted for publication January 4, 2016.

*Correspondence: ekomives@ucsd.edu

Morten Beck Trelle's present address is Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark.

Editor: Elizabeth Rhoades.

© 2016 by the Biophysical Society

0006-3495/16/02/0887/9



$I\kappa B\alpha$. Hydrogen-deuterium exchange MS (HDXMS) revealed intrinsic disorder in the fifth and sixth ARs (9), and showed that this region of $I\kappa B\alpha$ folds upon binding (10). NMR studies also suggested a folding-upon-binding process because although crosspeaks for AR5-AR6 were largely absent from spectra of the free protein (11), they could be observed in the bound complex despite its much larger size (12). These NMR studies also revealed that upon NF κ B binding, crosspeaks in AR3 weakened, suggesting a transfer of flexibility between the AR5-AR6 region and AR3 upon complex formation (12). AR6 in free $I\kappa B\alpha$ was shown by smFRET studies to fluctuate on long timescales, and these fluctuations were completely dampened by stabilizing mutations as well as upon NF κ B binding (13).

By placing the donor and acceptor fluorophores at various positions, Lamboy et al. (14) were able to obtain information about the flexibility of each repeat with respect to others, and the results revealed that AR1 fluctuates in both the free and NF κ B-bound states, whereas AR2 and AR4 never appear to fluctuate. Fluctuations were observed when FRET pairs were placed at AR3 and AR6, but since fluctuations were also observed when the FRET pairs were placed at AR2 and AR6, these fluctuations were attributed to fluctuation of AR6 (14).

In the initial smFRET study (14), an unexplained phenomenon was observed for $I\kappa B\alpha$ molecules labeled at AR3 and AR6. The FRET efficiency of the high-FRET ensemble actually decreased from 0.74 to 0.67 upon NF κ B binding (Fig. 1, B and C). This was an intriguing result because although one would have expected the increased foldedness of the NF κ B-bound $I\kappa B\alpha$ ARD (10) to result in a higher FRET efficiency, the opposite was observed. Assuming free rotation of the donor and acceptor fluorophore, this FRET efficiency corresponds to an increased distance of 3.3 Å between the labels upon NF κ B binding. The increased distance involving AR3 is consistent with the weakening NMR peaks in AR3 upon NF κ B binding, and might result from some part of AR3 cracking or becoming more dynamic in the bound state (15). On the other hand, AR3 is part of the most tightly folded segment (AR2-AR3-AR4) of the $I\kappa B\alpha$ ARD, and cracking at this position would reveal a dramatic change in the folding energy distribution of the domain. To investigate this phenomenon further, we decided to perform confocal-based smFRET studies to sample shorter measurement timescales than are accessible by total internal reflection fluorescence (TIRF). These studies, which used different donor and acceptor dye pairs, and were carried out in solution rather than on immobilized protein, again showed apparent ARD stretching. To investigate the role of NF κ B in this phenomenon, we compared $I\kappa B\alpha$ molecules in complex with either full-length NF κ B or molecules containing only the dimerization domains (NF κ Bdd). Deletion of the NTDs results in a modest 10-fold weakening of the binding affinity from 40 to 400 pM, but still allows formation of a tightly bound complex (4). HDXMS experiments

in which the effects of binding either full-length NF κ B or NF κ Bdd were compared revealed only decreases in amide exchange throughout the ARD of $I\kappa B\alpha$. Simulations recapitulated the results of the smFRET and HDXMS experiments, suggesting that binding of NF κ B induces a subtly different twist in the $I\kappa B\alpha$ ARD.

MATERIALS AND METHODS

$I\kappa B\alpha$ purification and labeling for FRET

All seven cysteines in $I\kappa B\alpha$ were replaced with serines by site-directed mutagenesis before the introduction of cysteines at positions 128 and 262 (for AR2-6), 166 and 262 (for AR3-6A), or 156 and 262 (for AR3-6B) as previously described (14). Expression and purification of the $I\kappa B\alpha$ constructs were performed as described previously (9) except that expression was induced with 0.2 mM isopropyl β -D-1-thiogalactopyranoside for 16 h. Immediately before labeling, $I\kappa B\alpha$ was passed through a size-exclusion column (S75; GE Biosciences) in labeling buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA) and concentrated in a centrifugal filter unit (Vivaspin; Sartorius) to 50–100 μ M. The detergent 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate was added to a 5-mM final concentration to reduce protein aggregation. Dimethyl sulfoxide was added to a 10% (v/v) final concentration to increase dye solubility during the reaction. Tris(2-carboxyethyl)phosphine was added to a 100 μ M final concentration.

In previous TIRF-based smFRET studies, the $I\kappa B\alpha$ was labeled with Alexa Fluor 555 (Thermo Fisher catalog number A20346) and Alexa Fluor 647 (Thermo Fisher catalog number A20347; $R_0 = 51$ Å). For the confocal-based smFRET studies presented here, Alexa Fluor 488 and 594 dyes (Thermo Fisher catalog numbers A10254 and A10256, respectively; $R_0 = 60$ Å) were used for compatibility with the laser and filter setup. In addition, a new sequential labeling protocol was developed to achieve higher labeling efficiencies. First, maleimide-conjugated Alexa 488 (donor fluorophore; Life Technologies) was dissolved in anhydrous dimethyl sulfoxide to a concentration of 1.5 mM and added to the protein solution to give a molar ratio of fluorophore/protein of 1:2. This substoichiometric labeling ratio was to minimize protein molecules with two donor fluorophores attached. The reaction mixture was protected from light and incubated at room temperature for 15 min. Mono-labeled protein was separated from unlabeled and double-labeled protein on a MonoQ 5/50 GL column (GE Healthcare) using a gradient of 0–100% buffer B in 40 column volumes at a flow rate of 1 mL/min (buffer A: 25 mM HEPES, pH 7.2, 50 mM NaCl, 1 mM EDTA; buffer B: 25 mM HEPES, pH 7.2, 1 M NaCl, 1 mM EDTA). The mono-labeled fraction was reconcentrated to 50–100 μ M and labeled as in the first reaction, but this time with maleimide-conjugated Alexa 594 (acceptor fluorophore; Life Technologies) at a fluorophore/protein ratio of 2:1. Unreacted fluorophore was removed using a PD10 gel filtration column (GE Healthcare) equilibrated in 25 mM HEPES, pH 7.2, 50 mM NaCl, 1 mM EDTA. Fluorophore and protein concentrations were estimated by spectrophotometry at 280 nm ($I\kappa B\alpha$, $\epsilon_{I\kappa B\alpha} = 12950$ cm⁻¹ M⁻¹), 488 nm (donor, $\epsilon_{A488} = 69000$ cm⁻¹ M⁻¹), and 594 nm (acceptor $\epsilon_{A594} = 73000$ cm⁻¹ M⁻¹). The absorbance at 280 nm was corrected for donor leakage of 11% and acceptor leakage of 56%. Finally, the labeled protein was purified on an S75 gel filtration column (GE Healthcare) in 25 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA to remove any aggregates that formed during labeling.

To determine whether the attached dyes were freely rotating, we performed steady-state anisotropy experiments on each $I\kappa B\alpha$ protein labeled at position 128 or 262 (AR2-6), position 166 or 262 (AR3-6), or position 156 or 262 (AR3-6B) with either the donor or acceptor purified as described above. Experiments were performed on the free and NF κ B-bound $I\kappa B\alpha$ proteins. The average anisotropy for the Alexa Fluor 488-labeled proteins was 0.1 (ranging from 0.07 to 0.13 for the different label positions).

The average anisotropy for the Alexa Fluor 594-labeled proteins was 0.18 (ranging from 0.13 to 0.19 for the different label positions). No significant differences were observed for the NF κ B-bound versus free I κ B α s. These values (all under 0.2) indicate free rotation of the dye under all experimental conditions. The emission intensity was also measured for the I κ B α protein labeled at position 128 or 262 (AR2–6). The fluorescence intensity of the free protein did not change upon addition of either a 1.2 \times or 10 \times concentration of NF κ B, indicating that the quantum yields were not affected by NF κ B binding.

NF κ B purification

The N-terminal hexahistidine-NF κ B (His6-p50_{39–350}/RelA_{190–321}) full-length heterodimer was coexpressed as described previously (12) and purified by nickel affinity chromatography (Ni-NTA agarose; Qiagen, Valencia, CA), cation-exchange chromatography (Mono S column; GE Healthcare), and size-exclusion chromatography (S200; GE Healthcare). The protein concentration was determined by spectrophotometry ($\epsilon_{\text{NF}\kappa\text{B}} = 43,760 \text{ M}^{-1} \text{ cm}^{-1}$). The N-terminal hexahistidine-NF κ B (p50_{248–350}/His6-RelA_{190–321}) dimerization-domain heterodimer was expressed and purified in a similar manner, but with the cation-exchange chromatography omitted and the size-exclusion chromatography performed on an S75 column rather than an S200 column. The protein concentration was determined by spectrophotometry ($\epsilon_{\text{NF}\kappa\text{B-dd}} = 22,900 \text{ M}^{-1} \text{ cm}^{-1}$).

smFRET experiments by confocal microscopy

Labeled I κ B α was diluted to final concentrations of 250 pM in 25 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA in a Tween 20-coated microscopy cuvette in the presence or absence of 2500 pM NF κ B full-length or dimerization domains. Donor and acceptor fluorescence signals were recorded via simultaneous two-channel data collection with a binning time of 500 μ s, using an in-house-built confocal single-molecule setup as described previously (16). The leakage of donor emission into the acceptor channel (6%) and acceptor emission due to direct excitation (4%) were taken into account. A threshold of 40 counts (the sum of signals from the two channels) was used to separate background noise from single-molecule fluorescence signals. E_{FRET} values were calculated from the corrected donor (I_D) and acceptor (I_A) fluorescence intensities:

$$E_{\text{FRET}} = I_A / (I_A + \gamma I_D).$$

The value of γ was approximated to 1 on the basis of our previous measurements (16). FRET efficiency histograms were generated and the distributions were fitted to a Gaussian function using OriginPro 7.0 (OriginLab).

HDXMS measurements

HDXMS was performed as previously described (17,18) with some modifications, using a Waters Synapt G2Si system with H/DX technology (Waters, Milford, MA). For initial peptide identification, the starting protein concentration was 15 μ M, whereas for HDX experiments the protein concentration was 5 μ M. Exchange reactions were prepared using a LEAP H/DX PAL autosampler (Leap Technologies, Carrboro, NC). The exchange reaction was initiated when 5 μ L of protein (initial concentration of 5 μ M) was mixed with 55 μ L of deuterated buffer (25 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol). For experiments in the presence of NF κ Bs, a 1:1 molar ratio of I κ B α /NF κ B dimer was used. The proteins were allowed to equilibrate at 25 $^\circ$ C for 5 min before exchange reactions occurred (0–5 min deuteration times). Then, the exchange reactions were quenched for 2 min at 1 $^\circ$ C using an equal volume of a solution containing 2 M GndHCl and 1% formic acid. The quenched sample was injected into a 50 μ L sample loop, followed by rapid on-line pepsin digestion

at 15 $^\circ$ C using a custom-built column made with pepsin-agarose (Thermo Fischer Scientific, Rockford, IL). Peptic peptides were captured on a BEH C18 Vanguard Pre-column (Waters), separated by analytical chromatography at 1 $^\circ$ C (ACQUITY UPLC BEH C18, 1.7 μ m, 1.0 \times 50 mm; Waters) with a gradient of 7–95% acetonitrile in 7 min (where both mobile phases contained 0.2% formic acid), and peptides were electrosprayed into a Synapt G2-Si quadrupole time-of-flight mass spectrometer (Waters). The mass spectrometer was set to MS^E-ESI+ mode for initial peptide identification and to Mobility-TOF-ESI+ mode to collect H/DX data. The mass range was set to 200–2000 (m/z), scanning every 0.4 s. Infusion and scanning every 30.0 s of leu-enkephalin (m/z = 556.277) was used for continuous lock mass correction. Peptides were identified and the MS/MS fragments were scored using PLGS 3.0 software (Waters). Peptides with a score of >7 were selected for analysis if their mass accuracy was at least 3 ppm and they were present in at least two independent runs. Deuterium uptake was determined by calculating the shift in the centroids of the mass envelopes for each peptide compared with the undeuterated controls, using DynamX 3.0 software (Waters). The amount of deuteration was corrected for back-exchange (~34%) based on a full-deuteration control.

Molecular-dynamics simulations

All simulations were carried out using the LAMMPS simulation package (19), in which the associative memory, water-mediated, structure and energy model (AWSEM) force field, which is a transferable coarse-grained protein model, was implemented (20).

In the coarse-grained scheme of AWSEM, each amino acid residue of a protein is simplified so as to be described by three beads: C α , C β , and O atoms (glycine is an exception due to its lack of a C β). Assuming an ideal geometry for the peptide bond, the positions of the rest of the atoms in the backbone can be calculated, so, unlike a C α -only model, stereochemistry is quite accurate. The energy function of the standard AWSEM can be schematically written as follows:

$$V_{\text{AWSEM}} = V_{\text{backbone}} + V_{\text{non-backbone}} + V_{\text{FM}}. \quad (1)$$

In Eq. 1, V_{backbone} refers to the force field that maintains backbone geometries of protein chains, and $V_{\text{nonbackbone}}$ includes physically motivated potentials that reflect the protein's chemical and/or physical properties in the context of protein secondary and/or tertiary interactions. These two energy terms are completely transferable among different proteins. The term V_{FM} (where FM denotes fragment memory) uses the similarity in local sequence to encode local structural tendencies using structures of peptide fragments available in the Protein Data Bank (PDB). In the following, we shall describe the above energy terms in more detail. V_{backbone} consists of five terms ($V_{\text{backbone}} = V_{\text{con}} + V_{\text{chain}} + V_{\chi} + V_{\text{rama}} + V_{\text{excl}}$) that fix the connectivity of the chain (V_{con}), the bond angles around the C α atom (V_{chain}), the chirality for the correct orientations of the C β atoms (V_{χ}), the backbone dihedral angles (V_{rama} , Ramachandran potential), and the excluded-volume interaction (V_{excl}). Note that V_{con} and V_{chain} are functions of the distances constrained by a combination of harmonic potentials.

$V_{\text{nonbackbone}}$ includes three terms ($V_{\text{nonbackbone}} = V_{\text{contact}} + V_{\text{burial}} + V_{\text{helical}}$). V_{contact} refers to the contact interactions of a protein's tertiary fold. It is defined by specifying the C β -C β distances that are far apart in sequence. The contact potential includes both direct and water (or protein)-mediated interactions between the residues. V_{burial} is a nonadditive potential that considers the preference of a residue to be buried inside the protein or to be exposed at the protein surface, and this preference depends on the residue type. V_{helical} is associated with the propensity to form helical structure, which requires the formation of an explicit hydrogen bond between a carbonyl oxygen of residue i and an amide hydrogen of residue $i+4$ in the backbone. This helical propensity also depends on the residue type.

The use of V_{FM} biases local structure to resemble the local structures of protein fragments with closely related local sequence. This term is drawn

from a database of short fragments called the Fragment Memory Library. The strategy employed here has been used in various forms (21) and resembles the associative-memory Hamiltonians of neutral network models (22). The fragment memory term takes into account local steric effects of side-chain packing that are modulated by the local sequence of proteins. This form of associative memory term has been successfully combined with physically based interactions to provide de novo structure predictions of protein tertiary folds (20,23,24). In this study, we used only the information of the native protein itself to obtain the fragment memories, biasing/funneling the local energy landscape toward the crystal structure. Such single-memory AWSEM simulations pinpoint the effects of physical forces on the folding and binding landscapes. More detailed descriptions of all the energy terms mentioned above can be found in the original AWSEM study (20) and the supplemental information therein.

A model of the solution structure of the full-length NF κ B-I κ B α structure was obtained by overlaying and merging PDB files 1NFI (RelA, p50dd, and I κ B α), 1IKN (RelA, p50dd, and I κ B α), and 1LE9 (p50 NTD) (NF κ B). The simulation protocol was used to generate an ensemble of low-energy structures of each structure: free I κ B α and two NF κ B-I κ B α complexes (one with only dimerization domains for RelA and p50, and one with both NTD and dimerization domains for both proteins). We adopted a micro canonical ensemble for the system, using a Langevin thermostat to control the temperature. We started from the native structure and equilibrated it at 300 K over 6 million steps. This corresponds to $\sim 1 \mu$ s of physical time.

RESULTS

FRET efficiencies observed in I κ B α labeled at AR3 residue 166 and AR6 residue 262 by confocal detection recapitulate TIRF measurements

We previously measured smFRET efficiencies (E_{FRET}) in free and bound I κ B α labeled at AR3 residue 166 and AR6 residue 262 (AR3–6) with Alexa Fluor 555 and Alexa Fluor 647 fluorophores. Although $\sim 20\%$ of the single-molecule traces showed some fluctuation in the FRET signal, if we consider only the stable, high-FRET signal, the average E_{FRET} in the free protein was 0.74 (Fig. 1 B; see Table 2). For the protein labeled at AR2 residue 128 and AR6 residue 262, the E_{FRET} was 0.78 (14). Using the R_0 for this dye pair of 51 Å, an E_{FRET} of 0.74 translates to a distance of 42.8 Å, which is slightly longer than the distance between the AR3–6 cysteine sulfur atoms of 38.5 Å measured in the crystal structures of the NF κ B-bound I κ B α (7,8) of the complex. This slightly longer distance is expected due to the linkers between the fluorophores and the maleimide attachment sites, which is estimated to be 5 Å based on previous modeling of similar dyes with similar linkers (25). The measured distance of 42.8 Å is therefore very close to the predicted distance for the NF κ B-bound state of 43.5 Å (Table 1). The E_{FRET} of the I κ B α AR3–6 bound to NF κ Bdd was also measured by TIRF and found to be 0.67 (Fig. 1 C) (14), corresponding to a distance of 45.3 Å, which is ~ 2.5 Å longer than in the free protein.

To investigate this phenomenon further and to obtain more statistically significant data, we carried out confocal studies using a different dye pair and optimizing labeling efficiencies (see Materials and Methods). Whereas several hundred mol-

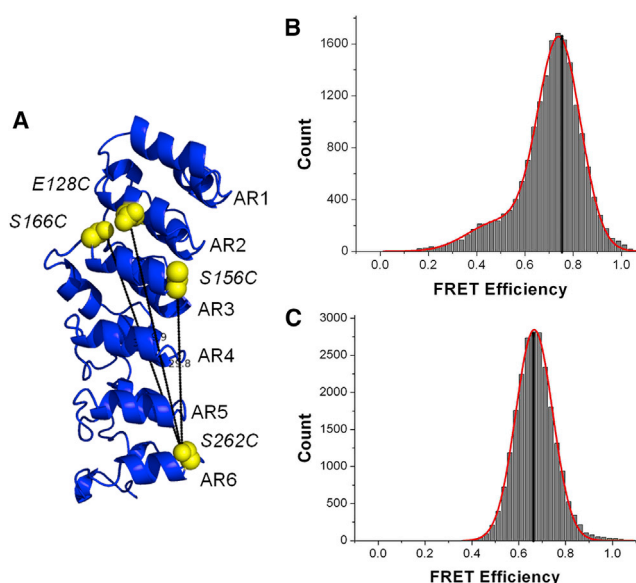


FIGURE 1 (A) Model of the structure of I κ B α (7,8) showing the various sites of cysteine substitution and labeling. (B) Histogram of FRET efficiencies measured by TIRF microscopy for free I κ B α labeled at positions 166 and 262. (C) Histogram of FRET efficiencies measured by TIRF microscopy for the same I κ B α labeled at positions 166 and 262 but bound to the dimerization-domain-only construct of NF κ B. To see this figure in color, go online.

ecules are typically measured using TIRF approaches, data from several thousand molecules are typically obtained in confocal-based measurements. Using the Alexa 488-Alexa 594 dye pair ($R_0 = 60$ Å), the E_{FRET} measured for the free I κ B α labeled at the same residues as before (residue 166 in AR3 and residue 262 in AR6 (AR3–6)) was 0.85 ± 0.03 , corresponding to a distance of 44.9 ± 1.7 Å (Fig. 2 A; Table 2). Upon binding of the dimerization-domain-only construct of NF κ B, E_{FRET} gradually decreased to 0.80 ± 0.05 , and it further decreased to 0.69 ± 0.03 upon binding of full-length NF κ B (Fig. 2, B and C; Table 2). Therefore, the distance between the FRET dye pair in I κ B α (AR3–6) increased from 44.9 Å to 52.5 ± 2.2 Å, an increase of 7.6 Å upon binding of full-length NF κ B.

In previous NMR experiments, we observed a weakening of crosspeaks for some residues in AR3 upon NF κ B binding (12). Since residue 166 was near the region where we observed weakening crosspeaks, we made an effort to label

TABLE 1 Distances and Expected FRET Efficiencies of Donor-Acceptor Pairs

Label Position	Label Position	Crystallographic Distance (Å) ^a	Expected E_{FRET} (TIRF)	Expected E_{FRET} (Confocal)
166 (AR3)	262 (AR6)	43.5	0.72	0.87
128 (AR2)	262 (AR6)	44.3	0.70	0.86
156 (AR3) ₂	262 (AR6)	34.8	0.91	0.96

^aThe crystallographic distance was calculated by measuring the distance between the side-chain sulfurs and then adding 5 Å according to information from Kalinin et al. (25).

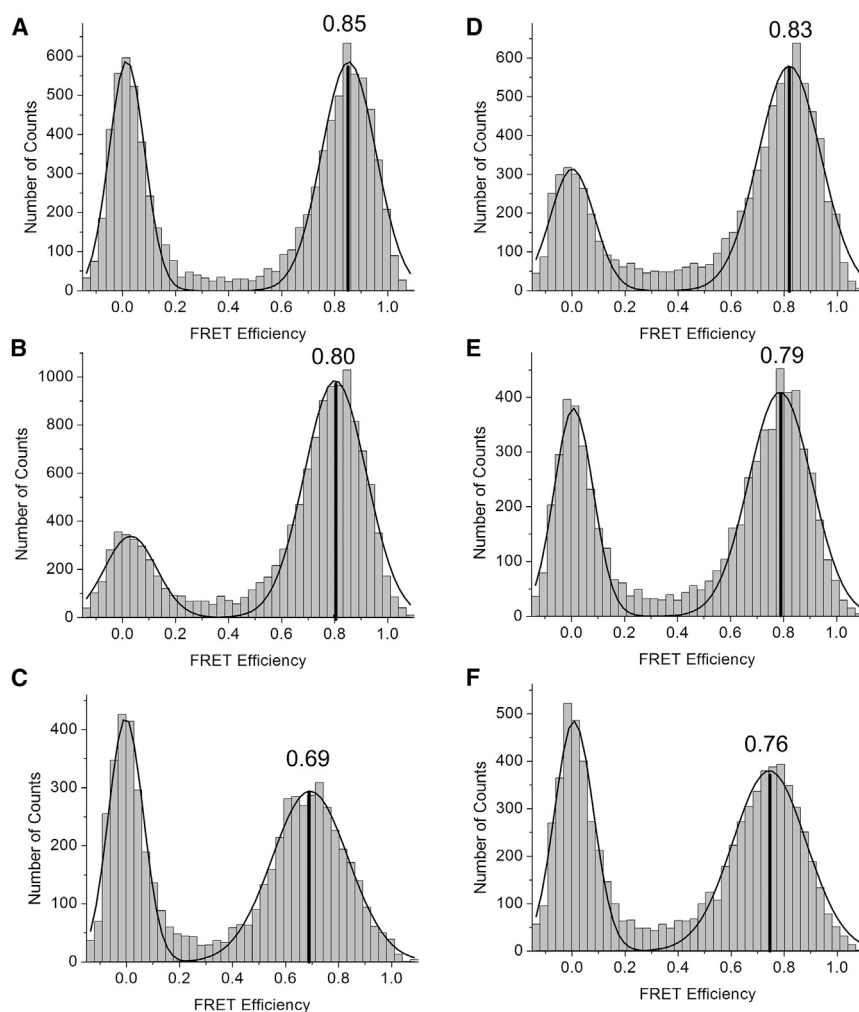


FIGURE 2 (A–F) FRET efficiency histograms determined by confocal microscopy for (A) free I κ B α labeled at 166 and 262 (AR3–6), (B) I κ B α (AR3–6) bound to dimerization-domain-only NF κ B, (C) I κ B α (AR3–6) bound to full-length NF κ B, (D) Free I κ B α labeled at 128 and 262 (AR2–6), (E) I κ B α (AR2–6) bound to dimerization-domain-only NF κ B, and (F) I κ B α (AR2–6) bound to full-length NF κ B.

AR3 at a different site. The only site that could be labeled resulting in stable protein was residue 156. The distance between this residue and residue 262 in AR6 was much shorter, only 29.8 Å. We completed a full confocal FRET study on this labeled protein, both bound and free; however, the observed E_{FRET} was always 0.95. Since the distance was so short, the FRET was, as expected, insensitive to small distance changes.

Comparison of I κ B α labeled at AR2 (residue 128) versus AR3 (residue 166) and AR6 (residue 262)

To determine whether this perceived stretching extended to AR2, we also measured the E_{FRET} of bound and free I κ B α labeled at AR2 (residue 128) and AR6 (residue 262). In this case, the E_{FRET} of the free I κ B α was 0.83 ± 0.01 (Fig. 2 D), corresponding to a distance of 46.0 ± 0.55 Å

TABLE 2 Distances from FRET Efficiencies

Label Position (Expected FRET)	TIRF or Confocal	Observed		Observed E_{FRET} (dd-Bound I κ B α)	dd-Bound I κ B α (Å)	Observed E_{FRET} (FL-Bound I κ B α)	FL-Bound I κ B α (Å)	Distance dd-Bound – Free (Å)	Distance FL-Bound – Free (Å)
		E_{FRET} (Free I κ B α)	FREE I κ B α (Å)						
AR3–6 0.72	TIRF	0.74	42.8	.67	45.3	ND	ND	2.5	ND
AR3–6 0.87	confocal	0.85 ± 0.03	44.9	$.80 \pm 0.05$	47.6	$.69 \pm 0.03$	52.5 ± 2.2	2.7	7.6
AR2–6 0.70	TIRF	0.78	41.7	0.76	42.1	ND	ND	0.4	ND
AR2–6 0.86	confocal	0.83 ± 0.01	46.0	0.79 ± 0.01	48.1	0.76 ± 0.02	49.5 ± 1.3	2.1	3.5
AR3–6B 0.96	confocal	0.94 ± 0.00	37.9	.95	36.7	.96	35.3	ND	ND

For TIRF, Alexa Fluor 555 and Alexa Fluor 647 ($R_0 = 51$ Å) were used. For confocal microscopy, Alexa Fluor 488 and Alexa Fluor 594 ($R_0 = 60$ Å) were used. Approximate distances were calculated from the E_{FRET} , taking into consideration the different R_0 values. Errors are standard errors of the mean. We performed four replicates for the free I κ B α samples, two replicates for the complexes with NF κ B(dd), and four replicates for the complexes with NF κ B(FL). Replicates were performed on different days and prepared from different labeled proteins. The expected distance was calculated from the NF κ B-bound I κ B α crystal structure (PDB ID: 1IKN).

(Fig. 2 A; Table 2). Again, we observed a gradual decrease in E_{FRET} (to 0.79 ± 0.01) upon binding of the dimerization-domain-only construct of NF κ B, and it further decreased (to 0.76 ± 0.02) upon binding of full-length NF κ B (Fig. 2, E and F). Therefore, the distance between the FRET dye pair in I κ B α (AR2–6) increased from 46.0 Å to 49.5 ± 1.3 Å, an increase of 3.5 Å upon binding of full-length NF κ B. Thus, an apparent stretching was also observed from AR2 to AR6, although it was not quite as pronounced as for AR3–6. We note that the TIRF measurements were only performed with the dimerization-domain-only construct of NF κ B, and no significant difference in E_{FRET} was previously reported for the AR2–6 labeled protein (13).

Observation by HDXMS of changes in amide exchange upon stretching of I κ B α

Given the weakening crosspeaks in AR3 observed by NMR upon NF κ B binding (12), we thought it likely that the perceived stretching was the result of dynamic local unfolding of some part of the I κ B α ARD. To test this hypothesis, we measured amide exchange in I κ B α using HDXMS. We previously reported HDXMS results for free and NF κ B-bound I κ B α detected by MALDI-TOF, but in those experiments we did not obtain coverage of key regions of AR3 (9,10). Here, using a Synapt G2Si instrument run in Mobility-TOF mode, we were able to achieve nearly 100%

sequence coverage (Fig. S1 in the Supporting Material). The data collected on the Synapt were completely consistent with previous results (Fig. S2) but also revealed subtle differences in exchange of NF κ B-bound I κ B α . In particular, residues 158–176 in AR3, residues 242–276 in AR6, and residues 278–287 (the PEST region) showed decreased exchange in the presence of the dimerization-domain-only constructs, but an even greater decrease in exchange in the presence of the full-length NF κ B (Fig. 3).

Molecular-dynamics simulations suggest two possible causes of the increased distance between residues 166 and 262: $d_{166-262}$

To probe the structural details of the NF κ B-I κ B α complex, we performed coarse-grained molecular-dynamics simulations using the AWSEM energy function, which is known to accurately predict protein monomer and dimer structures (20,26). The speed and accuracy of the coarse-grained model allow the widest exploration of conformational space, where twisting and/or stretching of the entire folded structure might not otherwise be visible due to the limited sampling available with all-atom approaches.

Consistent with the trend we observed in the experiments, the distance between the C β atoms of residues 166 and 262 ($d_{166-262}$) varies when the binding partner differs. The interaction between the dimerization domain of NF κ B and

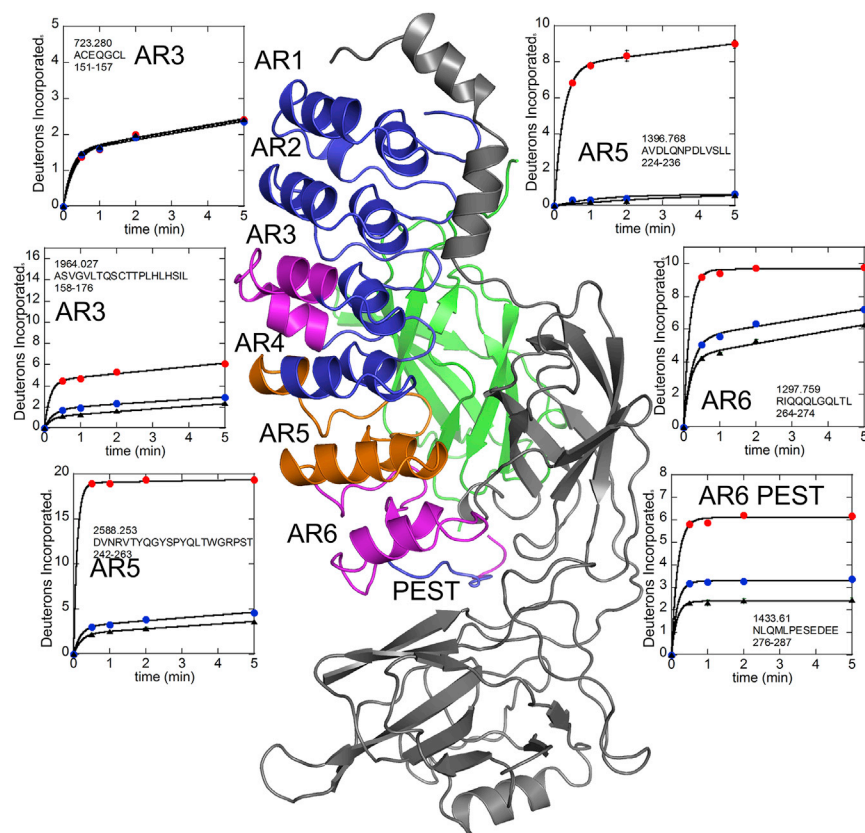


FIGURE 3 Deuterium-uptake plots for some regions of I κ B α . Residues 151–157 showed identical uptake in free I κ B α (red circles) and both NF κ B-bound forms (full-length (black triangles) and dimerization-domain-only (blue circles)). Residues 158–176 showed decreased amide exchange upon NF κ B binding, which was more pronounced upon binding of full-length NF κ B. Residues 224–236 (in AR5) showed decreased amide exchange upon NF κ B binding, which was not sensitive to the form of NF κ B. Several overlapping peptides encompassing the PEST region revealed that only the very C-terminal acidic residues sensed the form of NF κ B that was bound.

repeats 3–6 of I κ B α contributes partly to the increase of $d_{166-262}$. When I κ B α is bound to the NLS region but not tightly bound to the dimerization domain, the distributions of $d_{166-262}$ are almost identical to that of the free I κ B α (Fig. 4, *A* and *B*). When I κ B α is tightly bound to both the NLS and the dimerization domain (as assessed by $Q_{\text{interface}} > 0.6$), the mean distance between the residues to which the FRET dyes are attached becomes longer for I κ B α bound to the dimerization-domain-only NF κ B and even longer when it is bound to full-length NF κ B (Fig. 4, *C* and *D*). This further stretch is pronounced in the subset of molecules in which the PEST region is interacting with the NF κ B RelA NTD (Fig. 4, *E* and *F*).

DISCUSSION

Converting smFRET results into molecular distances is an inexact science, but progress is being made, particularly when the FRET dye pairs are attached to the macromolecule by relatively long linkers, and when relative distances are compared based on multiple attachment sites, as we did in this study. Here, we also compared measurements obtained by TIRF microscopy and confocal microscopy. For TIRF-based experiments, the molecules are immobilized (27) and generally fewer molecules are interrogated for longer periods of time. In confocal-based studies, the molecules remain in solution (28) and more molecules can be interrogated, but for much shorter times. Typically, better statistics can be obtained from confocal measurements (29). By comparing data from confocal and TIRF-based smFRET approaches, we were able to achieve improved confidence in our observations. Despite the use of different dye pairs, different solution conditions, and different microscopy-based approaches, a surprising decrease in E_{FRET} values be-

tween dye pairs in I κ B α labeled at AR3 and AR6, and to some extent also between AR2 and AR6, was observed upon NF κ B binding in every experiment. The observed decrease in E_{FRET} was consistent with an increased distance of >5 Å between the dye pairs upon full-length NF κ B binding. It is interesting to compare the distances calculated from measured E_{FRET} values with the distance between the labeling sites estimated from the crystal structures of the NF κ B-I κ B α complex (7,8). Estimates for the average distance between the dye and the labeling site were obtained for similar dyes with similar linkers via molecular-dynamics simulations (25,30). Surprisingly, the distance estimates obtained from the crystal structures were closer to the distances calculated from the E_{FRET} values measured in free I κ B α than to those measured in bound I κ B α . The distances measured in the I κ B α bound to full-length NF κ B were longer than what seemed possible based on the crystal structure. Thus, it appears that the I κ B α ARD adopts a structure that is more extended than that observed in the crystal structures. It is interesting to speculate that because the molecules were packed end-on-end in the crystals, crystal-packing forces may have compacted the structure. In addition, only the RelA NTD was present in the crystal structures. Indeed, molecular-dynamics simulations predicted that the most stable structure would have a different twist and bend of the ARD, resulting in a longer distance between the dye pairs than was calculated from the starting model based on crystal structures (7,8) (Fig. 4).

To examine whether the stretching involved local unfolding of AR3, as we previously predicted (12), we measured amide exchange in free and NF κ B-bound I κ B α . The HDXMS results showed no evidence of local unfolding of AR3. In fact, binding of NF κ B caused a decrease in amide exchange in AR3, and this decrease was more pronounced

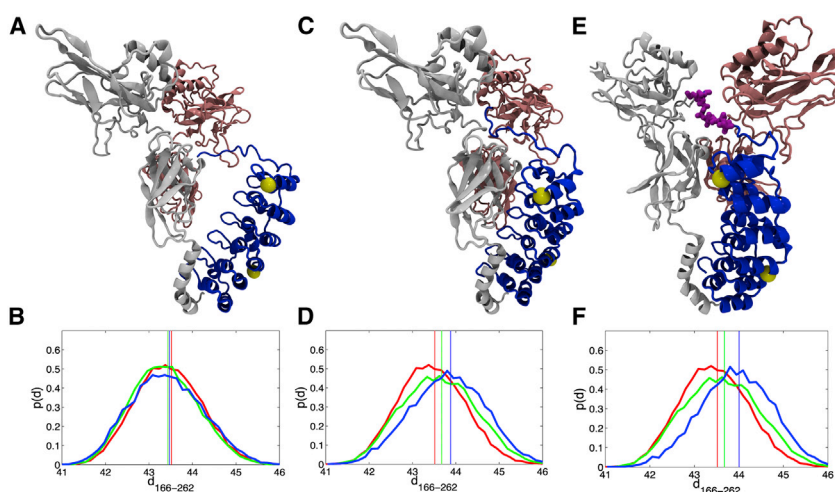


FIGURE 4 Representative structures from AWSEM simulations of the NF κ B-I κ B α complex. I κ B α , blue; RelA, light gray; p50 pink. The yellow spheres show the location of the C β atoms of residues 166 and 262. (A) Structures with $<60\%$ native interfacial contacts formed compared with the crystal structure were selected. (B) The probability distributions of the distance between the C β atoms of residues 166 and 262 were calculated from the subset of structures in (A) for free I κ B α (red), I κ B α bound to dimerization-domain-only NF κ B (green), and I κ B α bound to full-length NF κ B (blue), respectively. The vertical lines indicate the mean values of each distribution; $d_{166-262}$ was calculated by adding 5 Å to the measured distance between the C β atoms of residues 166 and 262. (C) Structures with $>60\%$ interfacial contacts formed were selected. (D) The probability distributions of $d_{166-262}$ for the subset of structures in (C) are plotted in three different colors according to the same scheme as in the previous panel. (E) Structures in which the PEST region (colored magenta) was interacting with RelA were selected. (F) The probability distributions of $d_{166-262}$ for the subset of structures in (E) are plotted in three different colors according to the same scheme as in the previous panel. The probability distribution for the subset of structures in which the PEST was interacting with the p50 NTD was almost identical to the green curve shown in (D).

to the green curve shown in (D).

upon binding of full-length NF κ B. Taken together, the results suggest that binding of NF κ B subtly alters the structure of I κ B α , perhaps causing a slight twisting or bending of the ARD to increase the distance between the N-terminal repeats (AR2 and AR3) and AR6. As previously reported, AR5 and AR6 showed dramatically decreased amide exchange upon binding of NF κ B (10). The decrease in amide exchange within AR5 was the same regardless of whether the NF κ B was full length or consisted of only the dimerization domains. In contrast, AR6 showed a larger decrease in amide exchange upon binding of full-length NF κ B as compared with the dimerization-domain-only construct (Fig. 3). We previously observed that mutations within I κ B α AR5 and AR6 result in chemical-shift changes in AR3 (11). Thus, it appears that the exact structure of AR3 is sensitive to the conformational state of AR5 and AR6. Indeed, our molecular-dynamics model of the lowest-energy conformation of NF κ B-bound I κ B α shows deviations from the starting structure mostly in AR3 and AR6.

The binding affinity of the complex between I κ B α and full-length NF κ B(RelA/p50) is extremely high: 40 pM at 37°C and immeasurably tight at 25°C (4). Nearly all of this binding affinity is due to interactions with the dimerization domains of the RelA/p50 heterodimer. Deletion of the NTDs weakens the binding to 400 pM (4). Even though the NTDs only contribute 10-fold to the binding affinity, they appear to contribute to the final bound structure of I κ B α as assessed by HDXMS data. We previously showed that I κ B α AR5 and AR6 have fully exchangeable amides in the free protein, but NF κ B binding dramatically decreases amide exchange, indicative of folding on binding (10). We now show that additional decreases in amide exchange in I κ B α AR3, AR5, and AR6 are observed when the NF κ B NTDs are present. Thus, amide exchange appears to be a sensitive readout for optimal folding of the I κ B α ARD. If we use amide exchange as a readout of protein foldedness, our results are consistent with the idea that optimal folding of the entire I κ B α ARD is only achieved upon binding of full-length NF κ B, which perhaps would explain why optimal binding affinity also requires full-length NF κ B. The notion that optimal folding throughout the I κ B α ARD is required for optimal binding is consistent with the hypothesis that the energy of folding the I κ B α ARD contributes to the very high binding energy of the complex (31).

According to the simulations, the optimal twist/stretch of the I κ B α ARD in the solution structure of the NF κ B-I κ B α complex appears to be attained upon interaction of AR3–6 with the dimerization domain, as well as upon interaction of the PEST region with the NTD of NF κ B RelA. Both interactions contribute to the final tightly bound structure, which recapitulates the observations from the smFRET data. NMR evidence suggested a direct interaction between the negatively charged DEE residues in the PEST region and the positively charged residues R30 and R33 in the RelA NTD (32). The configuration of the PEST region changes dramatically

between the starting model and the energy-optimized model of the NF κ B-I κ B α complex. It is likely that crystal-packing forces and the absence of the p50 NTD in the crystal structures both contribute to the fact that the PEST-NTD interaction was not observed crystallographically. We are pursuing a combination of molecular-dynamics simulations, cryo-electron microscopy, and small-angle x-ray scattering experiments to obtain a better understanding of the complete structure of the NF κ B-I κ B α complex in solution.

SUPPORTING MATERIAL

Two figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(16\)00007-2](http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)00007-2).

ACKNOWLEDGMENTS

The Waters Synapt G2Si instrument used in these experiments was provided by the NIH through project 1S100D016234-01. This work was supported by grants from the NIH (1P01-GM71862), NSF (grant MCB 1121959 to A.A.D.), and the Bullard-Welch Chair at Rice University (C-0016). T.C.L. was supported by a scholarship from the Kwanjeong Educational Foundation, South Korea. M.B.T. was additionally supported by grants from the Lundbeck Foundation (R18-A11217) and the Carlsberg Foundation (2012_01_0369).

REFERENCES

- Baeuerle, P. A., and D. Baltimore. 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell*. 53:211–217.
- Kumar, A., Y. Takada, ..., B. B. Aggarwal. 2004. Nuclear factor-kappaB: its role in health and disease. *J. Mol. Med.* 82:434–448.
- Hoffmann, A., and D. Baltimore. 2006. Circuitry of nuclear factor kappaB signaling. *Immunol. Rev.* 210:171–186.
- Bergqvist, S., C. H. Croy, ..., E. A. Komives. 2006. Thermodynamics reveal that helix four in the NLS of NF-kappaB p65 anchors IkappaBalpa, forming a very stable complex. *J. Mol. Biol.* 360:421–434.
- Baeuerle, P. A., and D. Baltimore. 1988. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science*. 242:540–546.
- Chen, F. E., D. B. Huang, ..., G. Ghosh. 1998. Crystal structure of p50/p65 heterodimer of transcription factor NF-kappaB bound to DNA. *Nature*. 391:410–413.
- Jacobs, M. D., and S. C. Harrison. 1998. Structure of an IkappaBalpa/NF-kappaB complex. *Cell*. 95:749–758.
- Huxford, T., D. B. Huang, ..., G. Ghosh. 1998. The crystal structure of the IkappaBalpa/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. *Cell*. 95:759–770.
- Croy, C. H., S. Bergqvist, ..., E. A. Komives. 2004. Biophysical characterization of the free IkappaBalpa ankyrin repeat domain in solution. *Protein Sci.* 13:1767–1777.
- Truhlar, S. M., J. W. Torpey, and E. A. Komives. 2006. Regions of IkappaBalpa that are critical for its inhibition of NF-kappaB-DNA interaction fold upon binding to NF-kappaB. *Proc. Natl. Acad. Sci. USA*. 103:18951–18956.
- Cervantes, C. F., L. D. Handley, ..., E. A. Komives. 2013. Long-range effects and functional consequences of stabilizing mutations in the ankyrin repeat domain of I κ B α . *J. Mol. Biol.* 425:902–913.
- Sue, S. C., C. Cervantes, ..., H. J. Dyson. 2008. Transfer of flexibility between ankyrin repeats in IkappaB* upon formation of the NF-kappaB complex. *J. Mol. Biol.* 380:917–931.

13. Lamboy, J. A., H. Kim, ..., E. A. Komives. 2011. Visualization of the nanospring dynamics of the I κ B α ankyrin repeat domain in real time. *Proc. Natl. Acad. Sci. USA*. 108:10178–10183.
14. Lamboy, J. A., H. Kim, ..., E. A. Komives. 2013. Single-molecule FRET reveals the native-state dynamics of the I κ B α ankyrin repeat domain. *J. Mol. Biol.* 425:2578–2590.
15. Ferreiro, D. U., A. M. Walczak, ..., P. G. Wolynes. 2008. The energy landscapes of repeat-containing proteins: topology, cooperativity, and the folding funnels of one-dimensional architectures. *PLOS Comput. Biol.* 4:e1000070.
16. Ferreon, A. C., Y. Gambin, ..., A. A. Deniz. 2009. Interplay of alpha-synuclein binding and conformational switching probed by single-molecule fluorescence. *Proc. Natl. Acad. Sci. USA*. 106:5645–5650.
17. Wales, T. E., K. E. Fadgen, ..., J. R. Engen. 2008. High-speed and high-resolution UPLC separation at zero degrees Celsius. *Anal. Chem.* 80:6815–6820.
18. Dembinski, H., K. Wismer, ..., E. A. Komives. 2014. Predicted disorder-to-order transition mutations in I κ B α disrupt function. *Phys. Chem. Chem. Phys.* 16:6480–6485.
19. Plimpton, S. 1995. Fast parallel algorithms for short-range molecular-dynamics. *J. Comput. Phys.* 117:1–19.
20. Davtyan, A., N. P. Schafer, ..., G. A. Papoian. 2012. AWSEM-MD: protein structure prediction using coarse-grained physical potentials and bioinformatically based local structure biasing. *J. Phys. Chem. B*. 116:8494–8503.
21. Friedrichs, M. S., and P. G. Wolynes. 1989. Toward protein tertiary structure recognition by means of associative memory Hamiltonians. *Science*. 246:371–373.
22. Hopfield, J. J. 1984. Neurons with graded response have collective computational properties like those of two-state neurons. *Proc. Natl. Acad. Sci. USA*. 81:3088–3092.
23. Papoian, G. A., J. Ulander, ..., P. G. Wolynes. 2004. Water in protein structure prediction. *Proc. Natl. Acad. Sci. USA*. 101:3352–3357.
24. Hegler, J. A., J. Lätzer, ..., P. G. Wolynes. 2009. Restriction versus guidance in protein structure prediction. *Proc. Natl. Acad. Sci. USA*. 106:15302–15307.
25. Kalinin, S., T. Peulen, ..., C. A. Seidel. 2012. A toolkit and benchmark study for FRET-restrained high-precision structural modeling. *Nat. Methods*. 9:1218–1225.
26. Zheng, W., N. P. Schafer, ..., P. G. Wolynes. 2012. Predictive energy landscapes for protein-protein association. *Proc. Natl. Acad. Sci. USA*. 109:19244–19249.
27. Ha, T., T. Enderle, ..., S. Weiss. 1996. Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor. *Proc. Natl. Acad. Sci. USA*. 93:6264–6268.
28. Deniz, A. A., M. Dahan, ..., P. G. Schultz. 1999. Single-pair fluorescence resonance energy transfer on freely diffusing molecules: observation of Förster distance dependence and subpopulations. *Proc. Natl. Acad. Sci. USA*. 96:3670–3675.
29. Deniz, A. A., T. A. Laurence, ..., S. Weiss. 2000. Single-molecule protein folding: diffusion fluorescence resonance energy transfer studies of the denaturation of chymotrypsin inhibitor 2. *Proc. Natl. Acad. Sci. USA*. 97:5179–5184.
30. Sakon, J. J., and K. R. Weninger. 2010. Detecting the conformation of individual proteins in live cells. *Nat. Methods*. 7:203–205.
31. Ferreiro, D. U., and E. A. Komives. 2010. Molecular mechanisms of system control of NF-kappaB signaling by I κ B α . *Biochemistry*. 49:1560–1567.
32. Sue, S. C., and H. J. Dyson. 2009. Interaction of the I κ B α C-terminal PEST sequence with NF-kappaB: insights into the inhibition of NF-kappaB DNA binding by I κ B α . *J. Mol. Biol.* 388:824–838.