Biophysical characterization of the free I κ B α ankyrin repeat domain in solution

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(RECEIVED March 15, 2004; FINAL REVISION April 16, 2004; ACCEPTED April 19, 2004)

Abstract

The crystal structure of I κ B α in complex with the transcription factor, nuclear factor κ -B (NF- κ B) shows six ankyrin repeats, which are all ordered. Electron density was not observed for most of the residues within the PEST sequence, although it is required for high-affinity binding. To characterize the folded state of I κ B α (67–317) when it is not in complex with NF- κ B, we have carried out circular dichroism (CD) spectroscopy, 8-anilino-1-naphthalenesulphonic acid (ANS) binding, differential scanning calorimetry, and amide hydrogen/deuterium exchange experiments. The CD spectrum shows the presence of helical structure, consistent with other ankyrin repeat proteins. The large amount of ANS-binding and amide exchange suggest that the protein may have molten globule character. The amide exchange experiments show that the third ankyrin repeat is the most compact, the second and fourth repeats are somewhat less compact, and the first and sixth repeats are solvent exposed. The PEST extension is also highly solvent accessible. I κ B α unfolds with a T_m of 42°C, and forms a soluble aggregate that sequesters helical and variable loop parts of the first, fourth, and sixth repeats and the PEST extension. The second and third repeats, which conform most closely to a consensus for stable ankyrin repeats, appear to remain outside of the aggregate. The ramifications of these observations for the biological function of I κ B α are discussed.

Keywords: protein folding; MALDI-TOF; amide H/²H exchange; ankyrin repeat domain; I κ B α ; functionally disordered proteins

The I κ B proteins regulate the activity of the Rel/NF- κ B transcription factor family. In quiescent cells, I κ B retains NF- κ B in the cytosol by masking the NF- κ B nuclear localization signal (NLS; Baeuerle and Baltimore 1988; Baldwin 1996). In response to a variety of extracellular stimuli, the I κ B protein undergoes phosphorylation-induced proteolytic degradation (Chen et al. 1995). Upon removal of I κ B, the NF κ B rapidly translocates to the nucleus, binds to specific gene promoters, and regulates gene transcription. Despite the extensive structural similarity between the I κ B family

members, I κ B α , I κ B β , and I κ B ϵ , they each respond differently to NF- κ B inducing signals as shown by their degradation rates and NF- κ B inhibition efficiencies (Baldwin 1996; Simeonidis et al. 1997; Tran et al. 1997; Ghosh et al. 1998; Hoffmann et al. 2002).

Sequence alignment showed I κ B α to be a member of the ankyrin repeat family (Bork 1993). The ankyrin-repeat domain (ARD) was first discovered as a repeated sequence in yeast cell-cycle regulation proteins (Breedon and Nasmyth 1987). It is named after ankyrin, a cytoskeletal adapter protein, which contains 24 tandem copies of the repeat (Michaely and Bennett 1993). Since first being discovered, over 2800 ankyrin repeat proteins have been identified, each containing between three and 24 copies of the ankyrin repeat (<https://coot.embl.heidelberg.de/SMART>) (Bork 1993). Ankyrin repeats are common in signaling proteins, and appear to be general protein–protein interaction motifs (Groves and

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.04731004>.

Barford 1999). Each ankyrin repeat is approximately 33 amino acids in length, and adopts a fold consisting of a β -hairpin followed by two antiparallel α -helices connected by a short loop. A variable loop connects each ankyrin repeat to the next. It is the α -helical stacks that are thought to form the small hydrophobic cores of the protein. The β -hairpin “fingers” projecting away from these helices, form the main protein–protein interaction sites (Yuan et al. 1999; Mosavi et al. 2002a). To date, there are nine high resolution structures of isolated ankyrin repeat-containing proteins, including multiple members of the INK4 family, myotrophin, and the I κ B family member Bcl-3 (Kriwacki et al. 1996; Tevelev et al. 1996; Luh et al. 1997; Baumgartner et al. 1998; Byeon et al. 1998; Venkataramani et al. 1998; Yang et al. 1998; Foord et al. 1999; Li et al. 1999; Zhang and Peng 2000; Michel et al. 2001; Zeeb et al. 2002). The only structural information about I κ B α comes from the I κ B α /NF- κ B complex structures (Huxford et al. 1998; Jacobs and Harrison 1998).

Recently, several structures of different classes of tandem repeat motifs have been elucidated including the armadillo, HEAT, leucine-rich repeats and tetratricopeptide families (Groves and Barford 1999). These tandem-repeat proteins assemble into elongated, modular, stacked arrays of regular repeating topology. This contrasts with the irregular topologies of globular proteins that are thought to be stabilized by numerous interactions between distant residues around a central hydrophobic core. The thermal stability of the ankyrin fold is thought to be brought about mainly by local interactions between adjacent structural units, and more than one repeat is required to form a stably folded ARD (Forrer et al. 2003; Kohl et al. 2003). Recently, stable ankyrin repeat proteins have been designed based on consensus sequences. These proteins are highly stable, and the design reveals key inter- and intrarepeat contacts that are thought to encode the heightened stability (Kohl et al. 2003). In addition, careful design strategies were used to optimize the “capping” of the first and last ankyrin repeats by mutating hydrophobic amino acids to arginines to promote solubility (Mosavi et al. 2002a; Forrer et al. 2003).

The crystal structure of I κ B α in complex with NF- κ B(p50/p65) shows that I κ B α interacts with NF- κ B via its central ankyrin repeat domain (Fig. 1). The surface area of interaction between the two proteins is extensive burying more than 4000 Å² (Huxford et al. 1998), and all six repeats are involved in formation of a noncontinuous contact surface. The first three ankyrin repeats of I κ B α contact the NLS polypeptide of p65, the fingers of the fourth, fifth, and sixth ankyrin repeats contact the dimerization domain of p50, the inner helices of the fifth and sixth ankyrin repeats contact the dimerization domain of p65, and the PEST region interacts with the N-terminal domain of p65. The crystal structure shows that I κ B α is well folded for the entire ankyrin repeat domain, and only the C terminus is disor-

Figure 1. Stereo view of the crystal structure of the NF- κ B(p50, p65)/I κ B α (67–317) complex. The NF- κ B p50 subunit is colored black and the NF- κ B p65 subunit is colored gray. Each of the six ankyrin repeats of I κ B α are colored separately; repeat 1, red; repeat 2, green; repeat 3, orange; repeat 4, blue; repeat 5, magenta; repeat 6, cyan; residues 281 and 282, purple. The electron density for residues 283–317 was not observed.

dered (Huxford et al. 1998; Jacobs and Harrison 1998). Yet, attempts to crystallize I κ B α were unsuccessful in the absence of the NF- κ B binding partner. To probe the folded state of the uncomplexed I κ B α , we present here results of both global indicators such as circular dichroism and calorimetry and local indicators such as amide H/²H exchange.

Results

Analysis of secondary structure and stability of I κ B α

Human I κ B α (67–317) was recombinantly expressed and purified as previously described (Huxford et al. 1998). The secondary structure of I κ B α was investigated by circular dichroism spectroscopy (CD). At 25°C, the CD spectrum showed a double minima at 208 nm and 222 nm and maximum at 195 nm, characteristic of α -helical proteins (Fig. 2A). Differential scanning calorimetry (DSC) experiments showed that I κ B α undergoes an unfolding transition with a midpoint (T_m) at $42 \pm 1^\circ\text{C}$ (Fig. 2B). The same T_m was obtained from the change in ellipticity at 222 nm as a function of temperature (data not shown). The same unfolding temperature was obtained under multiple buffers, salts, and protein concentrations; however, under all conditions tested it proved irreversible. The CD spectrum of I κ B α after incubation for 20 min at 55°C showed a loss of α -helical signal and formation of a signal that could be attributed to β -sheet-like structure (Fig. 2A). Size-exclusion chromatography confirmed that I κ B α incubated at 25°C is a stable monomeric protein, but incubation at temperatures above

Figure 2. (A) CD spectra of I κ B α at 25°C (black) and after incubation for 20 min at 55°C (gray). The spectra shown are samples of 10 μM I κ B α in 50 mM Tris buffer (pH 7.5). (B) DSC scan collected on 55 μM I κ B α under the same buffer conditions as the CD. The transition temperature was found to be $42 \pm 1^\circ\text{C}$. (C) CD spectra at 25°C of free I κ B α (filled circles), NF- κ B (filled squares), the NF- κ B complex (x), and the sum of the spectra of the two free proteins (open squares). The I κ B concentration was 16.5 μM , and NF- κ B(p50/p65 dimerization domains) was 33 μM . The plotted molar ellipticities take into account the difference in concentration between the two species. (D) The fluorescence emission spectrum of ANS binding recorded between 450 nm and 600 nm using an excitation wavelength of 360 nm. The emission spectra of I κ B α and ANS (filled circles) can be compared to controls of ANS alone (—), for NF- κ B alone (open diamonds). I κ B α in complex with NF- κ B still binds some ANS, but less than the free protein (x).

37°C for even 10 min causes complete conversion to the soluble aggregated form, which is completely excluded from the Superdex 75 column. Thus, conversion to the aggregate is the likely explanation for the irreversibility of the unfolding transition. In all the experiments presented here, care was taken to use only freshly purified monomeric protein kept below 25°C.

The mean-residue molar-ellipticity at 222 nm was $-14 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$ was similar to that observed for other ankyrin repeat proteins (Kriwacki et al. 1996; Tevelev et al. 1996; Luh et al. 1997; Byeon et al. 1998; Yang et al. 1998; Yuan et al. 1999; Zhang and Peng 2000; Mosavi et al. 2002b; Zeeb et al. 2002), but somewhat lower than might be expected based on the number of residues present in a helical conformation in the crystal structure of the I κ B α /NF- κ B complex (Huxford et al. 1998; Jacobs and Harrison 1998). Comparison of CD spectra of free I κ B α with I κ B α complexed to NF- κ B(p50/p65) dimerization domain showed

that no additional helical signal was observed upon complexation (Fig. 2C). Thus, all of the helical signal due to I κ B α in complex with the NF- κ B(p50/p65) dimerization domain is already formed in free I κ B α .

A diagnostic test used for proving disordered molten globule states is binding of 8-anilino-1-naphthalenesulphonic acid (ANS), which changes fluorescence intensity and emission wavelength upon binding molten globular proteins (Semisotnov et al. 1991). Figure 2D shows the fluorescence emission spectrum of the ANS complexes with I κ B α and NF- κ B(p50/p65) at equivalent concentrations. When ANS is added to I κ B α the emission spectrum of ANS undergoes a blue shift and a significant increase in intensity relative to ANS alone or ANS with NF- κ B(p50/p65). The strong ANS binding to I κ B α alone suggested that it may contain regions that have the characteristics of a molten globule. Studies on other molten globules show that helical CD signal is often still present in molten globular states, so it is possible that

I κ B α is molten globular (Ramboarina and Redfield 2003). In complex with NF- κ B(p50/p65), I κ B α still bound ANS, although less ANS binding was observed. This suggests that some portion of I κ B α may become more structured upon binding to NF- κ B.

Amide exchange to localize folded regions

Amide H/ 2 H exchange experiments were used to probe the solvent accessibility of different regions of I κ B α to assess which regions of the protein may be considered folded. Digestion of I κ B α with pepsin after various deuteration times resulted in 23 peptides from which quantifiable data could be obtained (Fig. 3A). The peptides covered 71% of the I κ B α sequence (Fig. 3B). This coverage included the C-terminal PEST and PEST extension sequences for which electron density was not observed in the crystal structures. It is interesting to note that because I κ B α is comprised of repeated sequences, similar pepsin fragmentation patterns are seen for several of the ankyrin repeats, allowing direct comparisons from one repeat to another to be made. Preliminary experiments showed that much of I κ B α became deuterated rapidly under physiological conditions, so a

flow-quench instrument (Kintek Corp.) was used to achieve short deuteration times.

Figure 4A shows how a typical mass envelope of a peptide shifts to higher mass as more labile amide positions exchange over time. Because the labeling occurs when the protein is in its native ensemble of states, the amount of deuteration reflects the solvent accessibility of a certain protein region. Comparing the mass spectra shown in Figure 4, A and C, it is clear that the region represented by the peptide of mass 1664.89 is less solvent accessible than the region represented by the peptide of mass 1374.77. The number of deuterons incorporated in each peptide was quantified by subtracting the centroid of the undeuterated control from the centroid of the isotopic peak cluster for the deuterated sample. Additional corrections for side-chain deuteration, and back-exchange after quenching were also made (Mandell et al. 1998b, 2001; Hughes et al. 2001). The number of amide deuterons incorporated was quantified for each peptide at each time point. Kinetic plots for two representative peptides are shown in Figure 4, B and D. The amount of exchange after 2 min at 25°C for 19 nonredundant peptides is given in Table 1.

Figure 4 shows the kinetic plots of amide exchange within the first (Fig. 5A–C) and sixth ankyrin repeats and the PEST sequence (Fig. 5D–F). The solvent accessibility of both of these end repeats was much higher than was observed for the middle repeats, and the PEST sequence was also nearly completely solvent accessible.

The kinetic plots of amide exchange within the second, third, and fourth ankyrin repeats of I κ B α are shown in Figure 6. The plots are organized according to the ankyrin repeat structure shown to the right of the plots so that in Figure 5, A–C corresponds to the β -hairpin finger regions, D–E corresponds to the helical regions, and F–H corresponds to the variable loop regions. Comparing the plots for these three middle ankyrin repeats, it can be seen that the β -hairpin finger of the third repeat is more solvent excluded than the same region of the second and fourth repeat. The β -hairpin finger of the third repeat has only one exchangeable amide out of 12, while there are two exchangeable amides out of 12 in the second repeat and two out of eight in the fourth repeat. Similarly, the helical region of the second repeat contains only two exchangeable amides out of 12, while there are nearly five out of 12 exchangeable amides in the helical region of the fourth repeat. Thus, there seems to be a trend that the third repeat is the most solvent excluded while the second repeat is slightly more solvent exposed and the fourth repeat is even more solvent exposed. Even the variable loop regions reflect this same trend, although they all showed at least 50% exchangeable amides.

Amide exchange experiments were also carried out on the soluble aggregated form of I κ B α prepared by incubation at 37°C for 10 min prior to the exchange reaction, which was also carried out at 37°C. If no aggregation had occurred, all

Figure 3. (A) MALDI-TOF mass spectrum of the peptides produced from I κ B α digestion with pepsin. (B) Sequence of I κ B α and a schematic of the repeated structural unit of the ankyrin repeat. The sequence is written ankyrin by ankyrin showing the location within the repeat unit of each peptide that was observed in the mass spectrum and that could be quantified for deuteration levels. The digestion conditions were optimized for maximum sequence coverage (see Materials and Methods), and the final coverage of the primary sequence of the protein was 71%.

Figure 4. (A) MALDI-TOF mass spectra of the peptide at m/z 1374.77 from the peptic digest of I κ B α after H 2 H exchange. The time evolution is (i) before deuterium, (ii) 0.1-sec deuterium, (iii) 2.5-sec deuterium, (iv) 10-sec deuterium, and (v) 60-sec deuterium. (B) The H 2 H exchange kinetic plot showing the number of amide deuterons the peptide at m/z 1374.77 incorporates over time. For all of the H 2 H exchange kinetic plots, the Y-axis maximum is the total number of amide positions in the peptide, and error bars represent the standard error of three independent determinations (most of the error bars are contained within the symbols). (C) The MALDI-TOF mass spectrum of peptide at m/z 1664.89 from the peptic digest of I κ B α . The deuterium periods are the same as those in A. (D) The H 2 H kinetic plot for the peptide at m/z 1664.89.

of the amides should have exchanged more rapidly due to the increase in temperature. On the other hand, aggregate formation would be expected to protect certain regions of the protein from exchange. The second and third finger regions exchange more deuterium at the higher temperature, and therefore, we can conclude that they do not participate in the aggregate (Fig. 7). The regions that become more solvent excluded in the soluble aggregate form include the helical region of the first ankyrin repeat, the variable loops and the PEST sequence.

Discussion

Assessment of the overall folded state of I κ B α . The thermal unfolding of I κ B α was followed by DSC and CD, and the results can be compared with similar data for other ankyrin repeat proteins. Both techniques, under a range of experimental conditions, showed that thermal melting was irre-

versible with a $T_m = 42 \pm 1^\circ\text{C}$. Although this T_m may appear low, it is similar to values reported for other ankyrin repeat proteins which range from 30°C for Notch to 53.1°C for myotrophin (Boice and Fairman 1996; Zhang and Peng 1996, 2000; Tang et al. 1999; Yuan et al. 1999; Zweifel and Barrick 2001; Mosavi et al. 2002a,b; Zeeb et al. 2002). Significantly higher thermal stabilities have only been reported for designed consensus ankyrin repeat proteins for which unfolding temperatures of 70 – 80°C have been reported (Mosavi et al. 2002a; Forrer et al. 2003; Kohl et al. 2003).

The CD spectrum of I κ B α reveals mostly helical secondary structure content, and the magnitude of the molar ellipticity at 222 nm is remarkably consistent with reports for various other ankyrin repeat proteins. In a CD study carried out on myotrophin, the α -helical content of 44% determined by CD was consistent with the value determined from the NMR structure of the protein (47%; Mosavi et al. 2002b).

Table 1. Summary of H^2H exchange data for $I\kappa B\alpha$

Region of $I\kappa B\alpha$	Peptide mass (m/z)	No. amides	No. exchanged 25°C ^a	No. exchanged 37°C ^a
ARD 1				
66 ^b –80	1761.86	14	10.2 ± 0.1	N/D ^c
79–91	1505.82	12	5.6 ± 0.1	4.6 ± 0.1
92–103	1374.77	11	6.3 ± 0.1	6.2 ± 0.1
93–103	1245.73	10	6.1 ± 0.1	6.2 ± 0.1
ARD2				
104–117	1679.89	12	1.4 ± 0.1	5.4 ± 0.1
105–117	1566.80	11	1.8 ± 0.1	N/D
ARD 2/3				
129–147	2001.98	16	9.2 ± 0.2	N/D
ARD 3				
137–150	1664.89	12	0.9 ± 0.1	5.1 ± 0.3
140–150	1325.71	9	1.2 ± 0.4	4.5 ± 0.1
142–150	1054.58	7	1.1 ± 0.2	2.5 ± 0.2
148–159	1343.64	12	2.2 ± 0.1	N/D
157–175	1964.02	16	7.9 ± 0.1	N/D
ARD 4				
176–186	1221.66	8	3.6 ± 0.1	4.0 ± 0.2
188–201	1521.84	13	3.2 ± 0.1	N/D
ARD 4/5				
201–220	2028.01	18	10.5 ± 0.2	9.7 ± 0.1
202–223	2278.15	20	12.5 ± 0.1	9.7 ± 0.1
ARD 6				
264–271	970.54	7	3.1 ± 0.1	3.0 ± 0.1
PEST				
296–317	2548.15	20	17.8 ± 0.2	14.8 ± 0.2
309–317	990.57	8	4.7 ± 0.2	4.3 ± 0.1

^a This value corresponds to the number of amide hydrogen positions that have exchanged after 120-sec incubation with deuterium. All values have been corrected for back-exchange, which was calculated to be 37% including the pepsin digestion step (see Materials and Methods).

^b Construct has an N-terminal Methionine residue.

^c N/D indicates that the deuteration of this peptide could not be quantified due to the poorer quality of the spectra obtained from protein incubated at 37°C.

For $I\kappa B\alpha$, the helical content as determined by CD was lower than predicted by the structure found in the crystal of the $I\kappa B\alpha$ /NF- κB complex, but the CD spectrum of free $I\kappa B\alpha$ is nearly identical to the CD spectrum of the $I\kappa B\alpha$ /NF- κB complex (Fig. 2C). Despite the fact that all of the helices appear to be formed in free $I\kappa B\alpha$, it shows significant ANS binding and rapid amide exchange over much of the protein. These findings suggest that the secondary structure of $I\kappa B\alpha$ is formed but the tertiary structure may not be compact. The observations for $I\kappa B\alpha$ including the T_m of 42°C, the helical CD spectrum, the rapid exchange of amides, and the large ANS binding are similar to observations made for the p16 INK protein (Boice and Fairman 1996). These authors concluded that p16 is highly dynamic but not molten globular due to its cooperative unfolding transition. Less but still significant ANS binding was observed when $I\kappa B\alpha$ was bound to NF- κB . This finding could be interpreted in two ways. One possibility is that even in the complex there are still regions of $I\kappa B\alpha$ that are not well struc-

tured. This is certainly possible given the somewhat high structure factors observed for $I\kappa B\alpha$ in the crystal (Huxford et al. 1998; Jacobs and Harrison 1998). Another possibility is that ANS binding will be a general property of such nonglobular proteins as ankyrin repeat domains, even when they are in complex with their binding partners.

The middle of the $I\kappa B\alpha$ ARD is most compact

The fact that similar peptide cleavages were obtained across several of the ankyrin repeats in $I\kappa B\alpha$ allow us to make comparisons between repeats. The third ankyrin repeat exchanges the least solvent deuterium. The second repeat exchanges somewhat more deuterium, and the fourth repeat exchanges even more. We were unable to collect data for much of the fifth repeat. The first and sixth ankyrin repeats of $I\kappa B\alpha$ are highly solvent exposed. If we posit that the solvent accessibility across these comparable regions indicates “compactness,” then we can say that the middle (third) ankyrin repeat is the most compact. The “compactness” then decreases slightly for the next two repeats moving out from the middle (the second and fourth) and the end repeats (the first and sixth) are the least compact. This observation contrasts with other ankyrin repeat proteins such as myotrophin and the INK family members, which are folded throughout the ARD (Tevelev et al. 1996; Kalus et al. 1997; Luh et al. 1997; Baumgartner et al. 1998; Byeon et al. 1998; Venkataramani et al. 1998; Yang et al. 1998; Li et al. 1999; Zhang and Peng 2000; Zeeb et al. 2002; Kohl et al. 2003). NMR relaxation studies of p16, p18, and p19 demonstrate that these proteins have very limited backbone conformational flexibility across all the ankyrin repeats (Kalus et al. 1997; Yuan et al. 1999).

One possibility is that the end sequences of $I\kappa B\alpha$ are not optimal for “capping” the $I\kappa B\alpha$ ARD. Indeed, one of the design parameters used in creating stable ankyrin repeat domains has been to introduce charged residues to “cap” the first and last ankyrin repeats to prevent aggregation (Mosavi et al. 2002a; Forrer et al. 2003). It is possible that for $I\kappa B\alpha$, it is NF- κB that provides the capping interactions. In the crystal structure of the complex, parts of NF- κB interact with both the first and sixth ankyrin repeats. These interactions are, in fact, with hydrophobic sequences in the first and sixth ankyrin repeats and in the PEST extension.

Aggregation of $I\kappa B\alpha$

$I\kappa B\alpha$ is prone to aggregation as are many ankyrin repeat proteins (Kalus et al. 1997; Yuan et al. 1999). We were able to use the amide exchange experiments to probe which parts of $I\kappa B\alpha$ are buried in the aggregate by comparing amide exchange rates at 37°C and 25°C. Even with no increase in protein mobility, the rate of base-catalyzed amide exchange is threefold more rapid at 37°C (Bai et al. 1993). Only two regions of $I\kappa B\alpha$ showed increased exchange at 37°C, and

Figure 5. The H^2/H exchange kinetic plots for the regions of I κ B α from the first and sixth ankyrin repeats and the PEST region. The Y-axis maximum is the total number of amide positions in the peptide. Deuterium incorporation was measured for residues (A) 67–80, the first β -hairpin; (B) residues 79–91, the helical region of the first repeat; (C) residues 92–103, the variable loop connecting the first and second repeats; (D) residues 264–271, the helical region of the sixth repeat; (E) residues 296–317, the PEST and PEST extension; (F) residues 309–317, the PEST extension.

although individual rate constants cannot be measured, the number of deuterons incorporated by 2 min was approximately threefold greater as expected. The two regions that showed increased exchange were the β -hairpin fingers in the second and third repeats. This result indicates that these two β -hairpin fingers were not buried in the aggregate. These are the only two β -hairpins in I κ B α that conform exactly to the TPLHL consensus sequence used in the design of highly thermostable ankyrin repeat proteins that did not aggregate (Fig. 7; Forrer et al. 2003). These folded β -hairpins, exposed on the outside of the aggregate, may be keeping the aggregate soluble.

The solvent accessibility of the helical region of the first ankyrin repeat decreases in the aggregate. The CD spectrum of the soluble aggregate shows a loss of α -helices consistent

with conversion of helical regions to β in the aggregate. It is possible that the other helical regions are also buried in the aggregate, but the quality of the mass spectra we were able to obtain from the aggregated protein preclude making this conclusion definitively. Finally, the C-terminal PEST and PEST extension also become buried in the aggregate. Thus, a regular pattern of burial and exposure was obtained, suggesting that the soluble aggregate has the first and sixth ankyrin repeats buried in the inside and the second and third exposed on the outside.

Physiological implications of the dynamic state of I κ B α

Free I κ B α displays a short half-life in the cell (Pando and Verma 2000). The half-life may result from the less than

Figure 6. The H^2/H exchange kinetic plots for the similar regions from the second, third, and fourth ankyrin repeats of IκBα. Deuterium incorporation was measured for residues (A) residues 104–117, the β-hairpin finger of the second repeat; (B) residues 137–150, the β-hairpin finger of the third repeat; (C) residues 176–186, the β-hairpin finger of the fourth repeat; (D) residues 148–159, the helices of the third repeat, (E) residues 188–201, the helices of the fourth repeat; (F) residues 129–147, the 2/3 variable loop and the third finger; (G) residues 157–175, the 3/4 variable loop; and (H) residues 201–223, the 4/5 variable loop and the fifth finger. A schematic of the secondary structure of an ankyrin repeat is shown to the right of the plots for reference.

completely well-folded character of free IκBα that we have found in this study. Second, a certain backbone malleability may allow IκBα to bind tightly and recognize multiple NF-κB targets (Kriwacki et al. 1996; Shoemaker et al. 2000). IκBα has similar binding affinities for several NF-κB partners, with a K_d of 6.0 nM for its primary cellular target p50–p65 NF-κB, 16.3 nM for the p65 homodimer, and 217.6 nM affinity for the p50 homodimer (Phelps et al. 2000).

The amide exchange results allow us to propose a model for recognition of NF-κB by IκBα. The second and third ankyrin repeat fingers, which appear to be rigidly folded, contain many of the contacts for the NF-κB nuclear localization signal (NLS; Jacobs and Harrison 1998). No electron density has been observed for the NLS in several crystal structures of NF-κB in the absence of IκB binding partners (Ghosh et al. 1995; Muller et al. 1995; Huang et al. 1997).

Conversely, the p50 dimerization domain of NF-κB, which is well-folded contacts the fifth and sixth ankyrin repeats of IκBα (Huxford et al. 1998). Thus, the IκBα/NF-κB interaction may involve a reciprocal folding upon binding mechanism in which the folded part of IκBα interacts with the unfolded NLS while the folded dimerization domains of NF-κB interact with the poorly ordered regions of IκBα. This reciprocal interaction may further stabilize IκBα by “capping” the ends of its ARD.

Materials and methods

Protein expression and purification

An IκBα expression plasmid was prepared by polymerase chain reaction (PCR) amplification of the region of MAD-3-cDNA encoding IκBα residues 67–317 and then ligated into a pET11a

Figure 7. Comparison of the amount of deuteration of the various regions of I κ B α after 120 sec at 25°C and 37°C. Regions that showed increased deuteration at 37°C are colored green; those that showed decreased deuteration at 37°C (indicative of protection due to aggregation) are colored red; those that did not change are colored gray; and those for which data could not be obtained at 37°C are dashed.

vector (Novagen) at the Nde I and BamH I restriction sites (Huxford et al. 1998). This portion of the I κ B α gene does not contain the signal response element, but does contain the PEST sequence and the PEST extension. The same fragment of I κ B α was used in the crystal structure determination of I κ B α with NF- κ B(p50/p65) (Huxford et al. 1998). The plasmid was transformed into the *Escherichia coli* strain BL21 (DE3) (Studier et al. 1990) and grown in minimal M9 media. Protein production was induced at room temperature with 0.1 mM IPTG for 14 h. The cells were collected by centrifugation, resuspended in 25 mM Tris (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 10 mM BME, and 0.5 mM PMSF, and lysed by sonication. The cell debris was removed by centrifugation and the soluble, crude lysate was loaded onto a Hi-Load Q-Sepharose (Amersham-Pharmacia) column. The final purification step was on a Superdex-75 gel filtration column (Amersham-Pharmacia). The purified protein was concentrated in a Centriprep-30 (Millipore/Amicon), and the final concentration was determined by both BCA assay (Pierce Biotechnology) and spectrophotometrically using a ϵ_{280} of 14650 M⁻¹ cm⁻¹. The protein concentrations determined by either method agreed to within 5%.

Circular dichroism (CD)

CD spectra were collected using an AVIV 202 instrument. CD spectra were acquired at 1–10 μ M protein concentration in a 0.02-cm pathlength cell. Samples of I κ B α were prepared in multiple buffering systems (all at 50 mM) including Tris, MOPS, and HEPES, at pH 7.5. The NaCl concentration was varied in MOPS, from 10 mM to 100 mM NaCl.

Differential scanning calorimetry (DSC)

DSC scans were collected using a MicroCal VP DSC instrument, at a concentration of 55 μ M using a scan rate of 90°C/h. Samples of I κ B α were prepared in multiple buffering systems including Tris, MOPS, and HEPES, at pHs between 6.0 and 7.5, or with urea

concentrations up to 3 M to explore the reversibility of the thermal unfolding transition.

Fluorescence

Fluorescence spectra were recorded on an ISA Instruments Fluoromax-2 spectroluminescencemeter in a 1-cm quartz cuvette at 25°C. The concentration of I κ B α was 10 μ M, and a final concentration of 20 μ M ANS was added to the protein. I κ B α was incubated with a twofold molar excess of NF- κ B prior to addition of ANS to determine the relative ANS binding of the I κ B α -NF- κ B complex. Emission spectra were recorded between 450 nm and 600 nm using an excitation wavelength of 360 nm.

Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF) spectra were acquired on a Voyager DE-STR instrument (Applied Biosystems) as previously described (Mandell et al. 1998a). The matrix used was 5.0 mg/mL α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich) dissolved in a solution containing a 1:1:1 mixture of acetonitrile, ethanol, and 0.1% TFA. The pH of the matrix was adjusted to pH 2.2 using 2% TFA. Slightly different coverage of the protein was obtained using a 1-min versus a 5-min pepsin digestion. The experiments were therefore analyzed with both 1-min and 5-min digestions.

I κ B α digestion and identification of digest products

Peptides produced by pepsin cleavage of I κ B α were identified by a combination of sequence searching for accurate masses, post-source decay sequencing, and Q-TOF MS/MS. To carry out the digest I κ B α was brought to pH 2.2 in a 0.1% TFA solution and then incubated with a nine-molar excess of immobilized pepsin for 1 min or 5 min. The monoisotopic mass (MH⁺) of all peptides identified was within 20 ppm of theoretical masses reported.

Amide H²H exchange experiments

The pH conditions during various stages of the reaction were determined on Accumet Inlab 423 pH electrode (Mettler-Toledo) using non-deuterated mock solutions (to avoid isotope effects with the electrode). The exchange reaction initiated when 130 μ M I κ B α buffered in 50 mM Tris (pH 7.7), 150 mM NaCl, and 1 mM dithiothreitol (DTT) was diluted 13.8-fold into D₂O. The protein was allowed to exchange (pD = 7.6) at room temperature for 0.1–300 sec. To capture the short times of deuteration, a RQF-3 flow quench apparatus (Kin-Tek Corp.) was used. The flow-quench experimental setup used two sample syringes, and a quench-vial kept incubated at 0°C placed at the end of the exit line. The deuteration periods collected were 0.1, 2.5, 5, 10, 20, 30, 60, 120, and 300 sec. After the exchange-period, the reaction was quenched into a 0°C solution of 2% TFA in H₂O (volume of 1190 μ L) to afford a sixfold dilution to a final solution of approximately 0.1% TFA (pH 2.2), and the same volume for all samples. The quenched protein then was digested as described above. The digest was aliquoted into several fractions, rapidly frozen in liquid N₂, and stored at –80°C.

To minimize back-exchange, samples analyzed by MALDI-TOF mass spectrometry were analyzed as previously described, and one sample was analyzed at a time (Mandell et al. 1998a). The I κ B α spectra were analyzed to determine the average number of

deuterons present on each peptic peptide. All values reported represent only the deuterons exchanged onto the backbone amide hydrogen (NH) position, all side-chain contribution due to residual deuterium (8%) were subtracted. Finally, data were corrected for back-exchange loss as determined as described previously (Mandell et al. 1998a; Hughes et al. 2001). For the experiments comparing exchange at 25°C versus 37°C, a 5-min pepsin digestion period instead of a 1-min digestion period was used. For the 1-min digest of the samples exchanged at 25°C, back-exchange was 24%; for the 5-min digest of samples exchanged at 25°C, back-exchange was 32%; and for the 5-min digest of samples exchanged at 37°C, back-exchange was 41%.

Acknowledgments

This work was funded by a grant from the NSF. C.H.C. acknowledges support from the Molecular Biophysics Training Program. We thank Diego Ferreira for helpful criticism of the manuscript.

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