Random coil conformation of a Gly/Ala-rich insert in IκBα excludes structural stabilization as the mechanism for protection against proteasomal degradation

Ainars Leonchiks[b,c], Edvards Liepinsh[a], Mikhail Barishev[c], Anatoly Sharipo[b,c], Maria G. Masucci[b], Gottfried Otting[a,*]

[a] *Department of Medical Biochemistry and Biophysics (MBB), Karolinska Institute, Doktorsringen 9A, S-171 77 Stockholm, Sweden*
[b] *Microbiology and Tumor Biology Center (MTC), Karolinska Institute, S-171 77 Stockholm, Sweden*
[c] *A. Kirchenstein Institute of Microbiology and Virology, Ratsupites 1, LV-1067 Riga, Latvia*

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Abstract Peptide segments of multiple glycine and alanine residues prevent the proteolytic degradation of ubiquitinated proteins by the proteasome. The structure of a Gly/Ala-rich insert in IκBα was probed by nuclear magnetic resonance (NMR) spectroscopy, comparing IκBα samples with and without Gly/Ala-rich insert. Narrow ^{1}H-NMR resonances at chemical shifts indicative of random coil conformations were observed in the difference spectrum. circular dichroism (CD) measurements further confirm that the mechanism of protection against proteolytic degradation is not based on structural transition or stabilization caused by the Gly/Ala-rich segment. In addition, most of the N- and C-terminal residues outside the ankyrin repeats in wild-type IκBα were found to be flexibly disordered.

Key words: Amino acid analysis; Gly/Ala-rich peptide; IκBα; Nuclear magnetic resonance; Random coil conformation

1. Introduction

Insertion of peptide segments of multiple glycine and alanine residues prevents the proteolytic degradation of ubiquitinated proteins by proteasomes [1]. This phenomenon is of medical importance, as a long Gly/Ala-rich segment protects the oncogenic Epstein-Barr virus (EBV) nuclear antigen 1 protein (EBNA1) against proteolysis [2,3]. More importantly, the available data suggest that Gly/Ala-rich segments may confer protection against proteasomal degradation to any protein, providing a simple means of extending the metabolic half-life time of peptide and protein drugs [1].

The mechanism of proteolytic protection by Gly/Ala-rich segments is not yet understood. One possible mechanism could be the stabilization of the proteins against unfolding, as unfolding is necessary to gain access to enzymatic sites in the catalytic core of the proteasome [4]. A structural role of Gly/Ala-rich segments is suggested by the fact that Gly-Ala repeats are found in proteins of stable secondary structure, such as spider silk and silkworm fibroins [5,6]. Quite generally, polypeptide chains of repetitive amino acid sequences can result in very stable structures, even when the individual amino acids have low propensities to occur in regular secondary structures, as illustrated by e.g. the collagen triple helix.

The protection effect of Gly/Ala-rich segments against proteolysis has been demonstrated in detail for the NF-κB inhibitor IκBα. Protection against proteolytic degradation was obtained with Gly/Ala-rich segments inserted at the N- and C-terminus or between residues Glu-55 and Pro-56, and a minimum insert of eight amino acid residues was found to be sufficient to elicit the protection effect [1].

The present nuclear magnetic resonance (NMR) study was initiated to address the question whether a Gly/Ala-rich segment of 26 amino acid residues between residues 55 and 56 (IκBα-GA24X) would have a specific, defined structure in solution. Two different constructs of IκBα were compared, with (IκBα-GA24X) and without (IκBα) the insert (Fig. 1). In addition, a 20-residue polypeptide containing exclusively glycine and alanine residues was investigated. As a spin-off of this study, information on the extent of structural disorder of the N- and C-terminal ends of IκBα was obtained.

2. Materials and methods

2.1. Materials

Plasmids expressing IκBα and the IκBα-GA24X chimera have been described previously [1]. IκBα and IκBα-GA24X expression plasmids were generated by cloning the corresponding coding sequences into the *Kpn*I site of the pQE30 expression vector (Qiagen) downstream of the 6 histidine tag. After ligation all the constructs were sequenced using an ALF DNA sequencer (Pharmacia).

The IκBα and IκBα-GA24X constructs were expressed in *Escherichia coli* strain JM109. Transformed bacteria were cultured in LB media containing ampicilin. At OD_{600} about 0.6–0.8 the culture cells were induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration 1 mM. Cells were harvested 6 h after induction, the bacterial pellet was washed in ice cold buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and subsequently lysed by addition of lysozyme to a final concentration 1 mg/ml for 30 min. The solution was sonicated at a high output setting (3–4 pulses, 20 s each) and centrifuged at 12 000×*g* for 20 min at 4°C.

The IκBα and IκBα-GA24X proteins were purified using the same protocol on Ni^{2+} cations immobilized His·Bind metal chelation resin 5-ml column affinity chromatography (IMAC) under non-denaturing conditions according to the manufacturer's recommendations (Novagen). The protein rich eluates were further purified on Bio-Gel HT Hydroxyapatite (Bio-Rad) columns equilibrated in 10 mM potassium

*Corresponding author. Fax: (46) (8) 335296.
E-mail: gottfried.otting@mbb.ki.se

Abbreviations: CD, circular dichroism; EBV, Epstein-Barr virus; EBNA1, EBV nuclear antigen 1; HSQC, heteronuclear single quantum coherence; IκBα, His_6-tagged construct of IκBα used here; IκBα-GA24X, IκBα with an Ala/Gly-rich insert of 26 residues between Glu-55 and Pro-56; INEPT, insensitive nuclei enhanced by polarization transfer; NMR, nuclear magnetic resonance; PEST domain, proline, glutamic acid, serine and threonine rich domain; TOCSY, total correlation spectroscopy; IPTG, isopropyl β-D-thiogalactopyranoside; IMAC, immobilized metal ion affinity chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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phosphate buffer, pH 7.3. The columns were washed with 10 bed volumes of 10 mM buffer and eluted with 35 mM buffer. Eluted protein fractions were collected and concentrated in Centricon-30 centrifugal concentrators (Amicon). The protein concentration was measured by the PCA assay (Pierce). The purity of the protein preparation was assessed by fractionation in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue R-250 staining [7]. From a 20-l culture of bacteria, approximately 20 mg of highly purified His_6-tagged IκBα protein and approximately 10 mg of highly purified His_6-tagged IκBα-GA24X protein were recovered, respectively.

The 20-residue peptide AGAGGGAGGAGAGGGAGGAG was purchased from Alta Bioscience.

2.2. CD spectroscopy

Circular dichroism (CD) spectra of IκBα and IκBα-GA24X were recorded at a protein concentration of 0.5 mg/ml in 5 mM potassium phosphate buffer, pH 7.3, and urea concentration ranging from 0 to 8 M. The CD spectra were recorded using a 1-mm pathlength demountable cell in an Aviv 62DS CD spectrometer at 25°C over the wavelength range of 200–240 nm with a step resolution of 1 nm.

2.3. NMR spectroscopy

NMR measurements were performed at 36°C with about 0.1 mM protein solutions in 90% H_2O/10% D_2O containing 10 mM sodium phosphate buffer and 25 mM glycerol at pH 7.1. 2D ^{1}H-NMR experiments were carried out on a Bruker DMX 600 NMR spectrometer. One-dimensional experiments were recorded with 256 scans with and without presaturation of the water resonance, using the jump-return [8] sequence with an interpulse delay of 100 μs for excitation. The non-uniformity of the excitation profile from the jump-return sequence was reversed by multiplication with the inverse excitation profile after Fourier transformation. Total correlation spectroscopy (TOCSY) [9] experiments were recorded without presaturation, using a clean MLEV-17 mixing sequence preceded and followed by 0.5-ms spin-lock pulses. The spin-lock pulse after the MLEV-17 sequence was followed by a delay of 100 μs and a spin-lock pulse of 2 ms duration applied with orthogonal phase to suppress the water resonance [10]. The TOCSY spectra were recorded with $t_{1max} = 54$ ms, $t_{2max} = 128$ ms, a mixing time of 50 ms and a total recording time of 4 h each. A gradient-enhanced ^{13}C-heteronuclear single quantum coherence (HSQC) spectrum [11] was recorded with a 0.4 mM solution of IκBα using $t_{1max} = 12.8$ ms, $t_{2max} = 157$ ms, relaxation delay of 55 and a total recording time of 2 days. Adiabatic 180° (^{13}C) inversion pulses of 300 ms duration were used in the insensitive nuclei enhanced by polarization transfer (INEPT) steps which were tuned to a $^1J_{HC}$ coupling constant of 140 Hz.

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-19                                    MRGSHHHHH HGSACELGTA
1     MFQAAERPQE WAMEGPRDGL KKERLLDDRH DSGLDSMKDE EYEQMVKELQ
51    EIRLE    GGGAGAGGAGGAGGAGAGGAGAGAVE  PQEVP RGSEPWKQQL
71    TEDGDSFLHL AIIHEEKALT MEVIROVKGD LAFLNFQNNL QQTPLHLAVI
121   TNQPEIAEAL LGAGCDPELR DFRGNTPLHL ACEQGCLASV GVLTQSCTTP
171   HLHSILKATN YNGHTCLHLA SIHGYLGIVE LLVSLGADVN AQEPCNGRTA
221   LHLAVDLQNP DLVSLLLKCG ADVNRVTYQG YSPYQLTWGR PSTRIQQQLG
271   QLTLENLQML PESEDEESYD TESEFTEFTE DELPYDDCVF GGQRLTL
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Fig. 1. Amino acid sequences of the IκBα and IκBα-GA24X constructs used in this work. The constructs were identical except for the Gly/Ala-rich insert (in bold). The starting methionine of the natural IκBα sequence is shown in bold and italics. The five putative ankyrin repeats are underlined. The PEST domain is shown in italics. Both constructs included an N-terminal His-tag with a few linker residues. The amino acids are numbered −19 to −1 for the N-terminal His-tag with linker residues, 1–55 for the residues before the Gly/Ala-rich insert, and 56–317 for the residues after the insert. The calculated molecular weights of the IκBα and IκBα-GA24X constructs are 37.6 and 39.4 kDa, respectively.

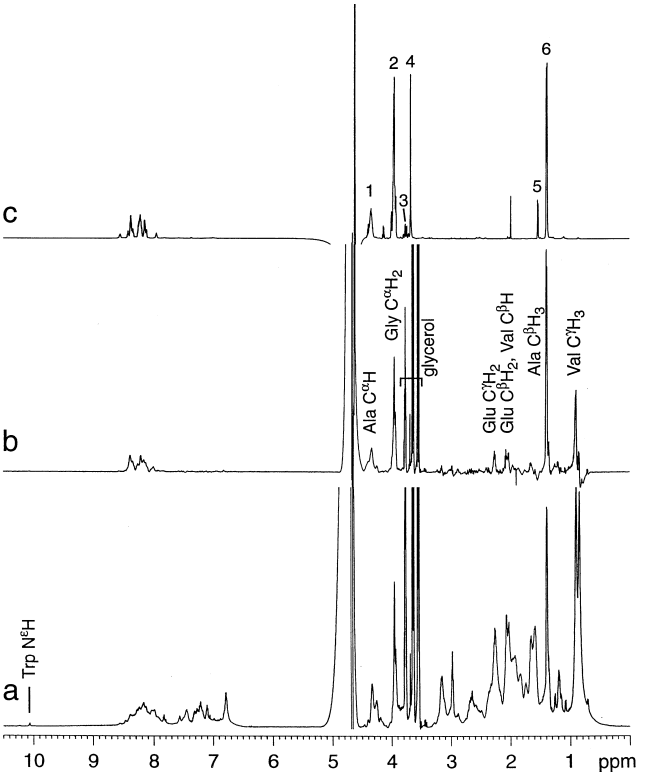

Fig. 2. Comparison of the difference ^{1}H-NMR spectrum of IκBα and IκBα-GA24X (both at pH 7.1) with the ^{1}H-NMR spectrum of a 20-residue Gly/Ala-rich peptide (pH 4.6) at 36°C. The spectra were calibrated with respect to the water signal at 4.7 ppm. a: ^{1}H-NMR spectrum of IκBα-GA24X. The narrow indol NH resonance of the flexible tryptophan residues is identified. A second, broader resonance is at 10.22 ppm. b: Difference between the ^{1}H-NMR spectra of IκBα-GA24X and IκBα. The signals from the Gly/Ala-rich insert, including the valine and glutamic acid residue at the C-terminus of the insert (Fig. 1), are identified together with the multiplets from the glycerol in the buffer. c: ^{1}H-NMR spectrum of the 20-mer AGAGGGAGGAGAGGGAGGAG. The resonances are assigned 1 = Ala $C^\alpha H$, 2 = Gly $C^\alpha H_2$, 3 = C-terminal Ala $C^\alpha H$, 4 = N-terminal Gly $C^\alpha H_2$, 5 = C-terminal Ala $C^\beta H_3$, 6 = Ala $C^\beta H_3$. The signal at 1.9 ppm stems from an impurity.

3. Results

3.1. Random coil conformation of the Gly/Ala-rich insert

One-dimensional ^{1}H-NMR spectra recorded of the IκBα (Fig. 2a) and IκBα-GA24X constructs were very similar, except that the spectrum of IκBα-GA24X contained some additional, narrow resonances. These could be individually observed by calculating the difference between the NMR spectra of IκBα-GA24X and IκBα (Fig. 2b). The resonances in the difference spectrum were much narrower than expected for proteins with molecular weight above 35 kDa, indicating that the Gly/Ala-rich insert is unstructured with pronounced mobility on the subnanosecond time scale. This conclusion is supported by the following observations: (i) the chemical shifts of the individual glycine and alanine residues of the insert are not resolved, indicating a very similar average chemical environment; (ii) the chemical shifts coincide closely with random coil chemical shifts [12], as can be seen by comparison with the ^{1}H-NMR spectrum of a related Gly/Ala-rich peptide (Fig. 2c); (iii) water presaturation almost completely bleached the amide proton signals (data not shown), indicating rapid exchange with the water as expected for a highly solvent ex-

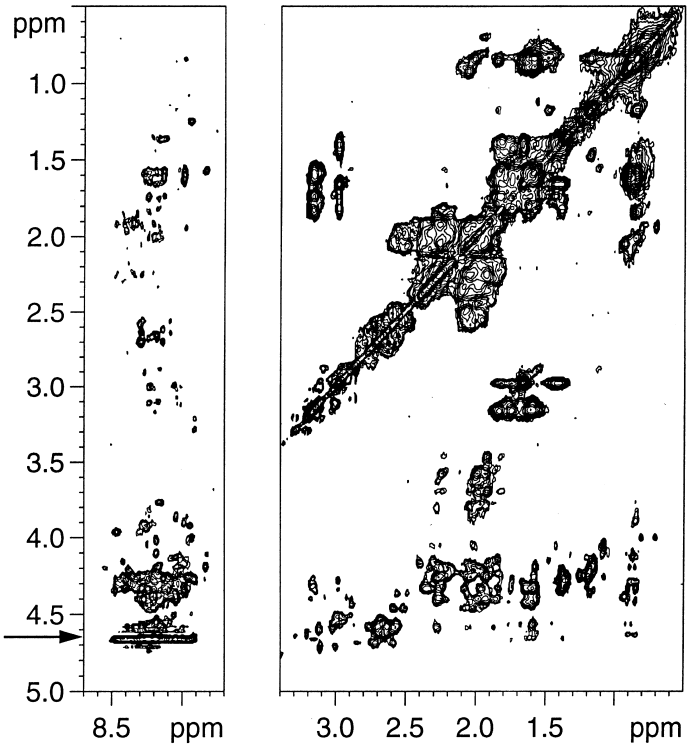

Fig. 3. Selected spectral regions from a TOCSY spectrum of IκBα, pH 7.1, 36°C, recorded without water presaturation. With the mixing time used (50 ms), only the narrow resonances from flexible parts of the molecule are observed. The F_1 frequency of the water resonance is identified by an arrow.

posed polypeptide. Besides the signals from the alanine and glycine residues of the insert, also the resonances of the valine residue of the insert could be identified in the difference spectrum of Fig. 2b. To confirm the resonance assignments in the difference spectrum of Fig. 2b, two-dimensional TOCSY spectra were recorded of IκBα and IκBα-GA24X. The difference spectrum showed the expected intraresidual connectivities (data not shown).

The flexible insert did not seem to change the conformation of the rest of IκBα. Significant changes in chemical shifts would have been expected from any major conformational change, with pronounced negative peak intensity in the difference spectrum as a result. This was not observed. The major peaks in the difference spectrum were positive and could be ascribed solely to the signals from the Gly/Ala-rich insert. Hence, the insert is not only unstructured, but it also acts like an independent domain.

3.2. Conformational stability

CD measurements performed at 25°C in the presence of different urea concentrations showed increased unfolding even at the lowest urea concentration used (1 M, data not shown). No significant difference in stability against urea denaturation was observed between IκBα and IκBα-GA24X. Therefore, the Gly/Ala-rich insert does not stabilize the conformation of IκBα.

3.3. Flexible segments in IκBα

The TOCSY spectrum of the IκBα sample without the insert revealed the presence of many amino acid residues with narrow ^{1}H-NMR resonances. Their amide proton resonances were between 7.7 and 8.6 ppm, as expected for unstructured polypeptide segments. Furthermore, the amide proton resonances gave rise to intense exchange cross peaks with the water signal, indicating high solvent exposure for those flexible residues (Fig. 3). The ankyrin repeats in the centre of the amino acid sequence probably present the rigid core of the protein (Fig. 1). Since NMR linewidths change gradually over the first three flexible residues at the boundary between a rigid domain and a flexible polypeptide segment [13,14], only larger segments with enhanced flexibility can give rise to the narrow linewidths characteristic of peptides with random coil conformation. The flexible segments are therefore expected to comprise the N- and C-terminal ends.

There are several lines of evidence suggesting that most of the 90 N-terminal and 69 C-terminal residues are flexible. First, two of the three tryptophan residues in IκBα are flexible, as the one-dimensional ^{1}H-NMR spectrum shows narrow (ca. 13 Hz linewidth) and broad (ca. 40 Hz) signals of tryptophan indol NHs at a ratio of 2:1 (Fig. 1). Second, there is at least one flexible isoleucine residue, as revealed by cross peaks with narrow linewidths in the ^{13}C-HSQC spectrum (Fig. 4). Third, there are cross peaks for all 20 amino acid residues in the ^{13}C-HSQC spectrum, all of them at random coil chemical shifts (Table 1).

3.4. Amino acid analysis of flexible polypeptides by ^{13}C-HSQC

Intensity measurements of ^{13}C-HSQC cross peaks may present a method for semi-quantitative analysis of the amino acid composition of flexible polypeptide segments. Since random coil conformations entail similar chemical environments, each cross peak contains the signal intensity from identical

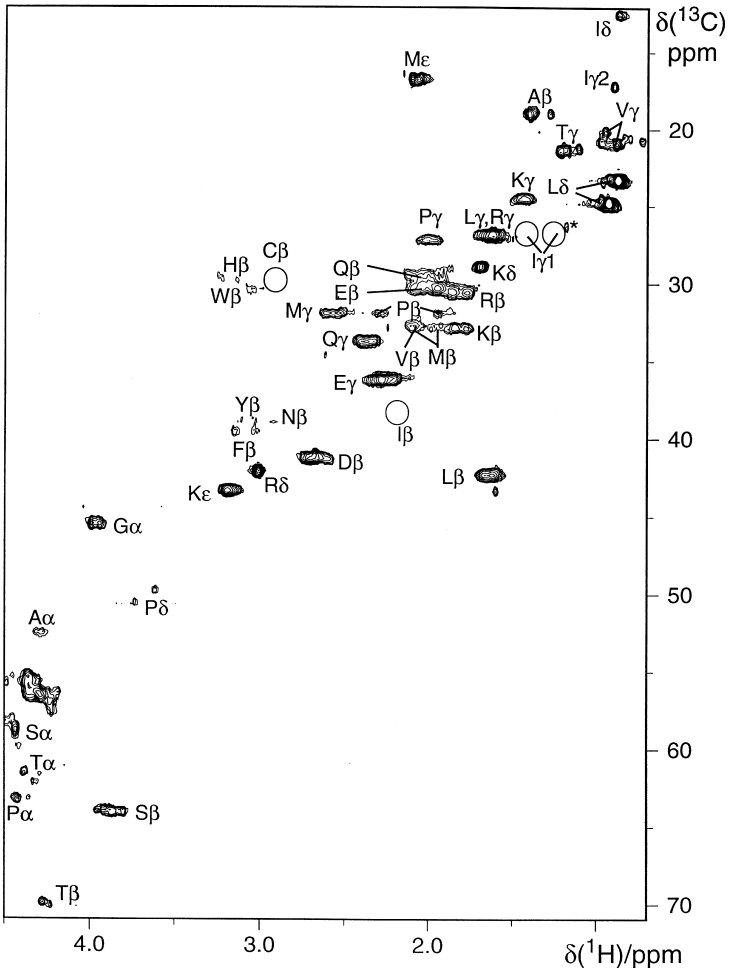

Fig. 4. Part of a ^{13}C-HSQC spectrum recorded with an 0.4 mM solution of IκBα, pH 7.1, 36°C. The cross peaks are labeled with the single letter amino acid code and the position of the respective CH group in the amino acid side chain. For example, Lβ identifies the cross peak of the $C^{\beta}H_2$ groups of leucine residues. Circles label the positions of cross peaks which were too weak to be observed at the plot level used. Stars identify cross peaks from low molecular weight impurities.

residues at different sequence positions. The contribution from residues in segments of defined three-dimensional conformation is relatively small, because their chemical shift distribution and relaxation rates are much larger. Since backbone resonances, including $C^{\delta}H_2$ groups of proline, show a significant, sequence dependent spread in chemical shifts in random coil polypeptides [15], side chain resonances should be used for the evaluation whenever possible. A major problem arises from the fact that proton relaxation rates and, consequently, cross peak intensities are sensitive to the time scale of mobility. While backbone relaxation rates tend to be uniform [13,14,16], increased mobility is usually observed towards the ends of amino acid side chains, with a concomitant increase in signal intensities of the corresponding ^{13}C-HSQC cross peaks. To minimize the bias from this effect, $C^{\beta}H_2$ resonances were used for the present analysis whenever possible.

Volume integration of the cross peaks yielded the amino acid distribution shown in Table 1. Using the isoleucine $C^{\beta}H$ cross peak as a reference and assuming that it originates from a single flexible isoleucine residue, the total cross peak intensity observed in the spectrum corresponds to over 80 flexibly disordered residues. This number presents a lower limit, because both isoleucine residues in the N- and C-terminal segments may be flexible.

Hardly any cross peak intensity was observed for the His-tag and the cross peaks from the aromatic residues and cysteines were weak. Since the pK_a of His side chains is near neutral pH and the protein samples were measured at pH 7.1, the cross peaks from the histidine residues may have been broadened beyond detection by chemical exchange between charged and uncharged side chains. Quite generally, $C^{\beta}H_2$ cross peaks from Tyr, Phe, Trp and Cys tend to be weak in ^{13}C-HSQC spectra of proteins. For the other amino acid residues, the error is estimated to be about 50%.

4. Discussion

The present data unambiguously show that a Gly/Ala-rich insert in IκBα assumes a flexible random coil conformation in solution and that its presence does not increase the stability of IκBα against denaturation. These results exclude a structural effect of the Gly/Ala-rich segment which might have been expected if the Gly/Ala-rich insert served as a physical protection against unfolding. Instead Gly/Ala-rich polypeptide segments may serve as signal peptides, protecting ubiquitinated proteins against proteasomal degradation by interaction with some yet unknown receptor.

Table 1
Amino acid analysis of the flexible segments detected in the ^{13}C-HSQC spectrum of IκBα

Amino acid	Number	Cross peak used	Amino acid	Number	Cross peak used
Glu	10.9	β	Thr	3.7 (4.0)	β (γ)
Asp	8.2	β	Pro	3.2 (3.1)	β (γ)
Leu	7.7	β	Val	2.6	γ
Gln	7.4	β	Phe	1.9	β
Arg	6.2	β	Tyr	1.5	β
Gly	4.7	α	Ile	1.0 (1.0, 1.0)	β (γ, δ)
Ala	3.7	β	Trp	0.9	β
Ser	4.2	β	Asn	0.9	β
Met	3.6 (4.5)	γ (ε)	Cys	0.5	β
Lys	3.6	β	His	n.o.	

The number of amino acids was derived from a volume integration of the ^{13}C-HSQC cross peaks, using the C$^{\beta}$H cross peak of Ile as reference. n.o.=not observed. Assuming that the Ile cross peaks stem from a single residue, the total sum of flexible amino acids would be 76.4.

The NMR analysis was technically straightforward, illustrating the power of NMR spectroscopy for discrimination between flexible and structurally rigid parts of a polypeptide chain. The sensitivity of the experiments was high, although the molecular weights of the proteins were too large for conventional sequence specific resonance assignments, the sample concentrations low by NMR standards and no isotope labeled samples were available.

The ^{13}C-HSQC experiment readily identified numerous flexible amino acid residues in the IκBα sample without insert, demonstrating that significant parts of the wild-type protein are disordered. IκBα contains five ankyrin repeats and a proline, glutamic acid, serine and threonine rich (PEST) domain (Fig. 1). The ankyrin repeats in IκBα (Fig. 1) probably present a rigid core structure, while secondary structure prediction algorithms predict disordered conformations for the PEST domain. Limited proteolysis experiments with chymotrypsin and V8 protease revealed cleavage sites at Trp-11, Leu-20, Glu-48, Glu-51, Trp-66, Leu-201, Tyr-251, Trp-258 and Glu-275, supporting the idea of increased flexibility outside the ankyrin repeat units, although Leu-201 is located in one of the presumably rigid ankyrin repeats [17].

There are no alanine or lysine residues among the 68 C-terminal residues and only a single methionine. On the other hand, there is only a single threonine among the 87 N-terminal residues. Combined with the NMR-derived information that two of the three tryptophans in IκBα are located in flexible segments and there is at least one flexible isoleucine and asparagine, a conservative estimate would predict flexibility for the N-terminus ending at Trp-66 and for the C-terminus starting from Asn-276, or for the N-terminus ending at Lys-38 and for the C-terminus starting from Trp-258. Flexibility of the polypeptide chain at Glu-55 in IκBα would explain the absence of major spectral changes caused by the Gly/Ala-rich insert in IκBα-GA24X as well as the apparent absence of any influence on the stability of the folded part of IκBα. Unambiguous determination of the flexible segments in IκBα will require shorter constructs with fewer flexible residues.

Attempts to crystallize the IκBα and IκBα-GA24X constructs used for the present study resulted in crystals which were too small for analysis by X-ray crystallography (A.L. and M.G.M., unpublished results). Since protein crystallization is more difficult when long segments of disordered polypeptide chains are present, it is likely that a smaller fragment of IκBα would crystallize more easily. Conceivably, NMR analyses as described here can be of great value to protein crystallography, since flexible polypeptide segments can readily be identified by NMR spectroscopy even when the molecular weight of the protein is beyond the limits for a structure determination by NMR.

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