Kinetics of NF-κB "stripping" by IκBα

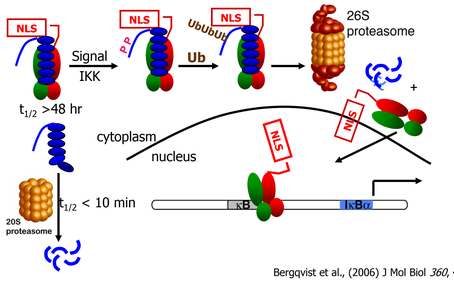
Vera Alverdi, Keith Cheung, Byron Hetrick, Simpson Joseph and Elizabeth A. Komives

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0378.

More than 150 target genes, involved in a wide variety of cellular functions, are regulated by the nuclear factor kappa B (NF-κB) transcription factors. NF-κB is induced by many classes of stimuli, and it plays a key role in the regulation of cellular development and proliferation and in the immune and inflammatory responses. Aberrant regulation of NF-κB has been implicated in a wide variety of disease states, including cancer, heart disease, AIDS, Alzheimer's disease, and arthritis.

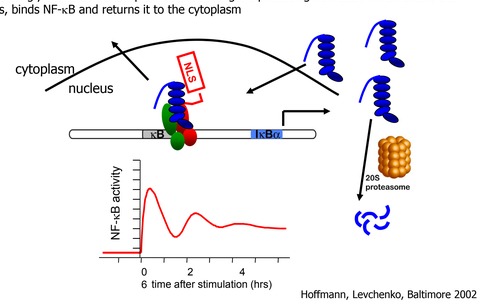
IκBα regulates NF-κB transcriptional activity

Cellular stress response causes IkBα phosphorylation, ubiquitination and degradation allowing NF-κB to enter the nucleus and activate transcription



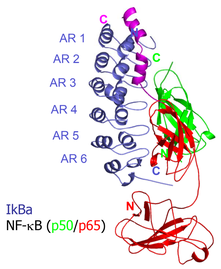
Bergqvist et al., (2006) J Mol Biol *360*, 421-34

NF-κB strongly activates transcription of the IκBα gene producing new IκBα which enters the nucleus, binds NF-κB and returns it to the cytoplasm



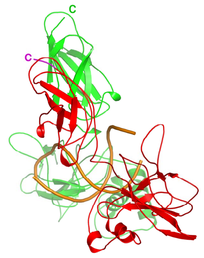
Hoffmann, Levchenko, Baltimore 2002

IκBα is an ankyrin repeat protein whose structure is known only in complex with NF-κB



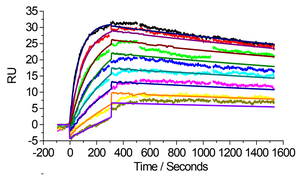
Huxford et al., Cell 1998

The crystal structure of IκBα (blue) bound to NF-κB (p50, green and p65, red; p65 NLS and flanking helices are magenta). IκBα contains six ankyrin repeats (AR), a ~33-amino-acid structural motif that generally adopts a helix-loop-helix-β-hairpin/loop fold. The p65 NLS and flanking helices (magenta) interact with the first three ankyrin repeats (ARs 1–3) of IκBα. The dimerization domains of both p50 and p65 form an extensive interface with ARs 3–6 and also form additional contacts with the C-terminal PEST sequence of IκBα. The p65 N-terminal domain also makes additional contacts with the C-terminal IκBα PEST sequence. The N-terminal domain of p50 is not involved in IκBα binding.

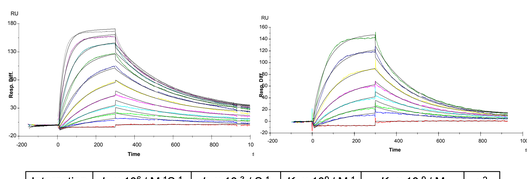


Chen *et al.*, Nature (1998) 391:410-13.

The crystal structure of DNA (yellow) bound to NF-κB (p50, green and p65, red). DNA contacts the loops protruding from the dimerization and N-terminal domains of the RHD and the linker between them.

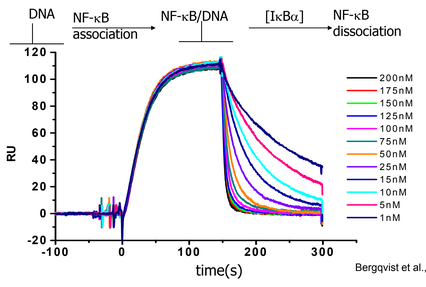


DNA	IκB	*ka* (x 10^6 $M^{-1}s^{-1}$)	*kd* (x $10^{-3}s^{-1}$)	K_D (nM)
IgκB	IκBα (67-317)	3.3 ± 0.6	0.14 ± 0.055	0.045± 0.01
IgκB	IκBα (67-287)	3.7 ± 0.1	0.15 ± 0.014	0.039± 0.003



Interaction	k_a x 10^6 / $M^{-1}S^{-1}$	k_d x 10^{-3} / S^{-1}	K_A x 10^9 / M^{-1}	K_D x 10^{-9} / M	χ^2
25	3.07 ± 0.13	3.2 ± 0.25	0.96 ± 0.04	1.0 ± 0.04	4.9
37	3.14 ± 0.41	4.69 ± 0.38	0.67 ± 0.05	1.6 ± 0.1	3.5

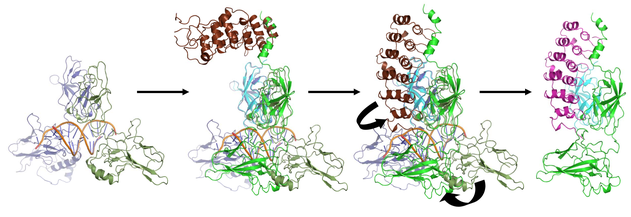
Kinetic enhancement of NF-κB : DNA dissociation by IκBα



$$R(t) = R_{max}(e^{-(kdDNA+kfd[I])t})$$

Bergqvist et al., (in preparation)

Model of IκBα-mediated kinetic enhancement of dissociation of NF-κB from DNA



NF-κB bound to DNA | Initial encounter at NLS | Conformational change and folding of ARs 5-6 | IκBα-NF-κB

How to prove the kinetics of this mechanism?

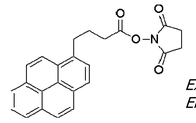
Fluorescence studies!!!!

DNA labeling and purification

The DNA used to bind NF-κB was the following hairpin sequence:

5'-AmMC6/GGGAAATTCCTCCCCCAGGAATTTCCC-3'

AmMC6 is an Amino modifier C6 group used to bind the fluorophore that in our experiments was the Pyrene succinimmide:



Ex: 343 nm
Emiss: 376 nm

1-Pyrenebutyric acid *N*-hydroxysuccinimide ester

Sean, Meth. Enz., 2007

The protocol used to label the DNA was:

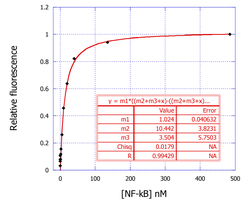
1. Prepared the following reaction mixture:
   - 75-uL of 0.1 M sodium tetraborate (Borax) (pH8.5)
   - 14-uL of a Pyrene succinimide solution made by dissolving 5-mg of 1-Pyrenebutyric acid n-hydroxyl succinimide ester (Aldrich) in 560 uL of DMSO
   - ~20,000 pmol (20 nmol) of DNA
   - Addition of H_2O to bring the reaction volume to 100 uL
2. Shake the mixture (use a vortex and set to low power) at room temperature for 6-hours
3. Add 1 mL of pure EtOH and store in -80°C for at least 30 minutes
4. Spin down the mixture
5. Re-suspend the pellet in 1 mL of mq H_2O
6. Purify the pyrene labeled DNA on HPLC using a RP-C18 column with a 60 min gradient from 0 to 60% buffer B (buffer A, ammonium acetate 20mM pH 6.5; buffer B, Acetonitrile).
7. Dry the sample overnight and re-suspend it in 100 uL of Tris 25 mM, NaCl 150 mM, EDTA 0.5 mM and DTT 1mM at pH 7.5 and 25°C.

Protein expression and purification

The p50(39-350)/p65(10-321) heterodimer of NF-κB were prepared using a coexpression method (Chen et al., 1999). A single expression vector contains the p50(39-350) and p65(10-321) genes, together with two ribosomal binding sites. A hexahistidine tag was added at the N-terminus of p50 (His-p50(39-350)). The heterodimer were purified with nickel affinity chromatography and Ionic exchange chromatography (cationic exchange resin, MonoS) followed finally by size exclusion on an S-200 column as previously described (Bergqvist et al., 2006).

Equilibrium binding constant

Titration measurements were performed using a photon-counting fluorometer (Fluormax-P, JY Horiba). All the samples were incubated at 25°C prior to start the experiments. K_D determination was performed with a constant pyrene labeled hairpin-DNA (pyrene-hairp-DNA) concentration in a 1 mL cuvette (5nM in 1 mL final volume). The NF-κB was titrated into the cuvette containing the pyrene-hairp-DNA. The sample was exited at 346 nm while the emission was monitored at 377 nM. The slits width for the emission and the excitation were 2 and 5 nm respectively with an integration time of 2s. A 3-minutes equilibration time was used between each titration measurement and 3 scans were taken at each point. A blank has been run by titrating correspondent amount of NF-κB into the tris buffer used during the experiments. The intensities fluorescence obtained were subtracted off the values in the presence of labeled DNA. The fitting program used was Kaleidagraph software 4.0.



K_d = 10.4 nM

For equilibrium, it is best to have labeled species at concentration below the K_d for the interaction.
The quadratic equation fits:

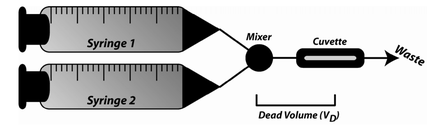
Y=m1*([m2+m3+X)-sqrt[(m2+m3+X)^2-4*m3*X])/(2*m3)

where Y correspond to the maximum fluorescence
m1 is the amplitude of fluorescence
m2 correspond to the K_D
m3 to the pyrene-hairp-DNA concentration and X to the [NF-κB]

$$Y = \frac{I_{max}\,(K_d + [R] + c) - ((K_d + [R] + c)^2 - 4[R]c)^{1/2}}{2[R]}$$

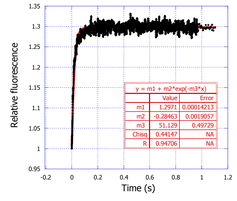
Stop flow fluorescence:

Rapid kinetic experiment was performed at 25°C on the uSFM-20 (Biologic, France) stopped-flow instrument. The mixing volume was 120 uL with sampling period at 200 us and 5 ms.



Association experiment

The association experiment for the pyrene-hairpin-dna was performed at 10 different concentration of NF-κB heterodimer (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1, 1.5, 3 uM) maintaining constant the concentration of the pyrene-hairp-DNA (0.1 uM before mixing). The experiment has been repeated at least 3 times. The association data have been elaborated using the fitting program Kaleidagraph 4.0.



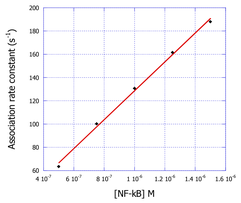
Syringe 1: Pyrene-hairp-DNA (0.25uM)

Syringe 2: NF-κB (different concentrations)

Y = m1 + m2*exp(-m3*x)

that can be clearly write as:

$RL_t = RL_{eq}\,(1-e^{-kobst})$



y = 4.5572 + 1.2422e+8x R= 0.99841

$k_{obs} = k_{off} + k_{on}[L]$

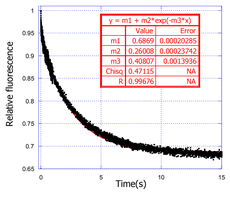
The association of NF-κB with DNA has a

k_a = 1.2 x 10^8 $M^{-1}s^{-1}$

Extrapolation should yield the k_d, 4.55 s^{-1}, but this is not very accurate because experiment is done above Kd.

Dissociation experiment

The dissociation experiment was performed fixing the concentration of NF-κB:hairp-DNA complex in ration 2:1 and adding an excess of unlabeled hairp-DNA (1:10, 1:50 and 1:100, complex:hairp-DNA). The same experiment was repeated using $IκBα_{67-287}$ instead of unlabeled hairp-DNA in the same concentrations and ratios. The data were elaborated and fitted with Kaleidagraph software 4.0 using the exponential decay equation.

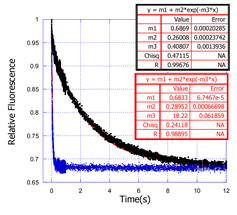


Syringe 1: Pyrene-hairp-DNA (0.25uM) / NF-κB (0.5uM)

Syringe 2: unlabeled hairp-DNA (10x, 50x, 100x more than NF-κB)

Y = m1 + m2*exp(-m3*x)

k_{off} = 0.41 s^{-1}



Syringe 1: Pyrene-hairp-DNA (0.25uM) / NF-κB (0.5uM)

Syringe 2: IκBα (10x, 50x, 100x more than NF-κB)

Literature

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