Different DNA Sequences Sense the Presence of the NF-κB TAD in Different Ways

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Abstract

The NF-κB family of transcription factors is responsible for the activation of hundreds of genes related to inflammation and the immune response, as well as other processes. In the cell, the proteins occur as homo- and heterodimers between the different members, the most common of which is the p50/p65 heterodimer. In previous in vitro studies, it was not possible to study the full length of this dimer, as the transactivation domain (TAD) of the p65 protein was cleaved off during protein expression. However, recent methods have made it possible to express the full length protein, prompting further study of the effects of the TAD on the NF-κB pathway. It is believed that the TAD plays a large role in the interactions between the proteins and the transcription site, however the nature of this role is unknown. The purpose of this experiment is to test for any differences in binding affinity between the short- and full-length proteins and various transcription sites, specifically those for urokinase and IFN-κB proteins.

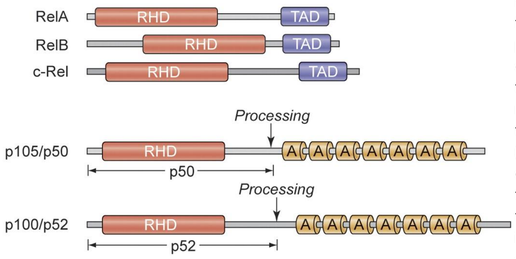

Fig 1: NF-κB Family. There are five proteins in the NF-κB family, however only three possess the TAD. In vivo, only dimers with a TAD will transcribe DNA, and as a result some dimers inhibit transcription. The p50/RelA (p50/p65) dimer is the most common in vivo, though in the same conditions it is also typically bound to an inhibitor, IκBα, when in the cytoplasm.

Protein Purification

For this experiment, we already possessed semi-purified full length proteins, however we needed to prepare new samples of short length protein from scratch. We began by transforming BL21 DE3 competent cells with a plasmid for producing NF-κB, and plated them onto an LB-AMP plate. After incubation, we used the cells to grow two 10ml M9ZB starter cultures, which were in turn used to inoculate two 1L M9 minimal media cultures. These cultures were allowed to grow until their optical density was between 0.6 and 0.8. We then removed the cultures and induced them with IPTG before leaving them to grow for 16 hours. At the end of this time, we spun down the samples and resuspended the pellet in a buffer containing imidazole, protease inhibitor, and βME. The solution was then sonicated to fragment the cells and then spun down for 45 minutes. We then put the supernatant through an Ni^{2+} column to separate HIS-tagged proteins. Proteins like NF-κB that possess a histidine tag will bind to the ions in the column, where they can be later eluted to purify them from the solution. After running the solution through the column, the eluted protein was dialyzed overnight with SEC buffer and then separated into 2ml aliquots to be stored at -80°C. For the experiment, we needed to further purify the samples from the aliquots. The samples, one with the TAD, and one without the TAD, were first run through a MonoS ion exchange chromatography column at 4°C. After collecting fractions and concentrating them back to 2ml, the samples were then run through a size exclusion column at 4°C and concentrated. To ensure correct purification, the samples were analyzed on an SDS PAGE gel before the experiment.

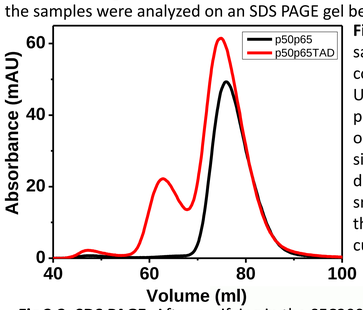

Fig 2.1: SEC200 Elution Profile. Both protein samples were separately run through an SEC column to separate out any other residual proteins. UV absorbance was used to monitor presence of protein in the column, and fraction collection began once the spike began. In the short length curve, the single peak in absorbance represents only the dimer. However, in the full length curve, the first, smaller peak is the larger full length protein, while the second, larger peak is the short length. In this curve, only fractions from the first spike were used.

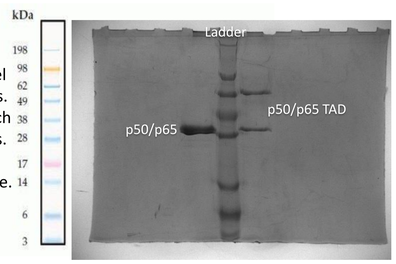

Fig 2.2: SDS PAGE. After purifying in the SEC200 column, small samples of each protein were run through sodium dodecyl sulfate–polyacrylamide gel electrophoresis to ensure the purity of the samples. When run through the gel, the dimers separate such that the p50 and p65 proteins form separate bands. For the short length protein, the p50 and the p65 are almost the same size, so only one band is visible. Meanwhile, for the full length protein both bands are visible as the TAD drastically increases the weight of the p65 protein.

DNA Labeling

The DNA samples were labeled with FITC and purified using HPLC with a C_{18} reverse phase column. The IFN-κB sample was already labeled and purified, while we labeled fresh urokinase promoter for the experiments. The column separated the sample into groups of lone dye, lone DNA, labeled DNA, and combinations of the three. The free DNA ran through the column first, followed by labelled DNA and then lone dye. After about 30 minutes, the UV reading spiked, and we began collection fractions. We determined that only fractions 7-14 contained DNA, and ran these fractions on 1% agarose gels to determine which had the largest concentrations. As seen on the gels below, fraction 8 had the largest concentration of labeled DNA, and was therefore chosen for our experiments.

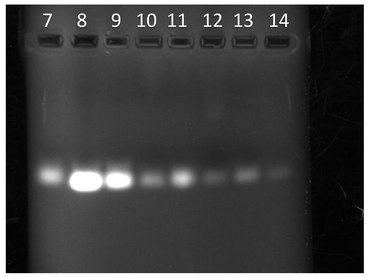

Fig 3.1: 1% Agarose EtBr Gel. Ethidium Bromide binds to the DNA and fluoresces when exposed to UV light. This gel confirms the presence of DNA in all the fractions.

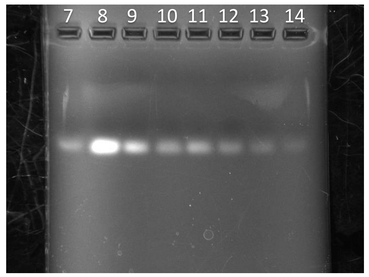

Fig 3.2: 1% Agarose Gel. The second gel lacks EtBr, and thus only the labeled DNA will fluoresce when exposed to light.

Fluorescence Anisotropy

The binding affinity of each sample was measured using fluorescence anisotropy. Anisotropy is a phenomenon wherein a property of a substance differs based on its orientation, or in the case of fluorescence, how fast it reorients. Due to Brownian motion, all molecules in a solution will spin, with larger molecules spinning slower. When exposed to polarized light, the molecule will react differently based on its orientation and spin in relation to the light, emitting a photon either in a parallel polarization to the original, or in a perpendicular one. Anisotropy can be used to calculate binding affinity as the unbound DNA will spin faster and emit light in a different polarization than the slower-spinning bound DNA.

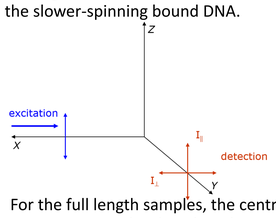

Fig 4.1: Principle of Fluorescence Anisotropy. The light emitted by the excitation system is polarized along the z-axis before reaching the samples. When the samples fluoresce, they emit light either along the z axis or the y, which is then detected in respect to a cartesian plane. If it is along the z axis, it is considered a parallel polarization. If it is along the y, it is perpendicular.

For the full length samples, the central concern was differences in affinity between different DNA sequences. For the short length, we were instead measuring differences in affinity compared to the full length. The samples were prepared by adding varying concentrations of protein to a set concentration of DNA (5 nM), as seen in the table below. Each full length sample was only measured once, to match it with previous results, while the samples without the TAD were measured in triplicate to ensure accurate results. The fluorescence anisotropy was measured in a microplate photometer, with each well containing 200µl of sample. In the machine, the samples were first illuminated with polarized light by an excitation system, causing them to fluoresce. This emitted light was then separated from the excitation light by an emission filter, and then detected and recorded.

[Protein] (nM)	Protein V (µl)	Buffer V (µl)
0	0	300
4	12	288
8	24	276
10	30	270
12	36	264
16	48	252
20	60	240
24	72	228
28	84	216
32	96	204
36	108	192
40	120	180

Fig 4.2: Microplate Samples. Samples were measured over a range of concentrations to account for differences in emission based on low or high concentration. The buffer used in preparing the samples was the SEC buffer from purification. In addition to the buffer and protein, 300µl of DNA was added to each sample..

Anisotropy Results

To find our Keq value, we first plotted our anisotropy results on scatter plots. The anisotropy value on the y axis is calculated from the mean polarization for each sample. From these anisotropy values we created a trend curve for the graph using the equation $y = A1*e^{-x/t1} + y0$. In this equation, x is concentration, y is resulting anisotropy, and t1 is the keq value for the interaction.

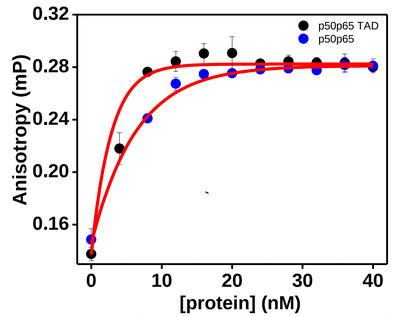

Fig 5.1: Urokinase promoter Anisotropy Graph.

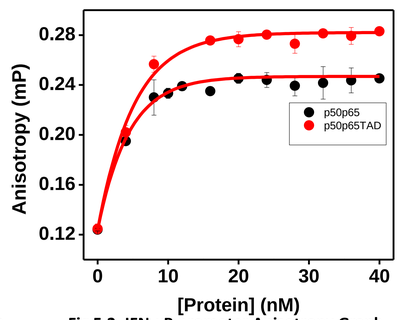

Fig 5.2: IFN-κB promoter Anisotropy Graph.

On both graphs, certain concentration values were removed due to an obvious deviation from the curve, most likely due to error. Both graphs plateau at a concentration of ~30nM, as all DNA has bound to protein.

Analysis and Conclusions

The anisotropy was significantly higher for the full length protein. While this is in part due to the larger size of the full length protein, it also indicates that the TAD plays a role in the protein binding to DNA, and prompts further research into the nature and extent of this role. Interestingly, on the IFN graph the plateaus for the two dimers are at separate anisotropies, while they are at almost the same for the urokinase promoter. This could be due to error, however it could also indicate vast differences in the way the full length protein binds to different transcription sites. For the urokinase promoter, the data suggests the full length protein binds tightly to the DNA, reducing the size to almost that of the short length. For IFN, however, it would appear the TAD does not tightly bind to the DNA, drastically increasing the complex's size compared to the short length. For the urokinase promoter, the Keq value for the full length protein is much smaller than the short length, as was expected, however for the IFN samples the full length possesses a slightly higher Keq value. This could be the result of error or some other factor, however the difference is too small to impact results. Ultimately, however, the Keq is only one variable for the Michaelis-Menten equation, Keq = (Ka)/(Kd + [S]), which outlines the binding affinity for the reaction.

Sample	Keq (µM)	Deviation
IFN-κB p50/p65	4.4	0.4
IFN-κB TAD	5.13087	0.32099
Urokinase p50/p65	6.35754	0.3751
Urokinase TAD	2.80374	0.34359

Looking Forward

The overall goal of these experiments is to study the differences in interaction between the short and full-length dimers and DNA. After calculating the binding affinity, Kd, the next step is to perform stopped-flow in order to study the kinetics of the binding reaction and calculate the interaction's association constant, ka. From there, the two values can be plugged into the Michaelis-Menten equation in order to calculate the dissociation constant for the interaction. Because of the differences between the two sets of data, it would also be a logical step to perform hydrogen deuterium exchange mass spectrometry, in order to find the exact differences between the bound forms of the full length protein. Finally, this process will need to be repeated with other sets of DNA, in order to ascertain other differences in the binding process.

References

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