

Quantifying Domain Specific p53/Bcl-2 Affinity

Summary

In order to study the interaction between the anti-apoptotic protein Bcl-2 and the tumor suppressor p53, nano-porous optical interferometry (nPOI) is used to identify which domain of p53 interacts with Bcl-2. After showing that the central-region of p53 is responsible for the interaction, the dissociation equilibrium constant (K_D) of the complex is measured without labeling the proteins. This new technique yields a K_D of 670 nM which compares favorably with the literature value of 535 nM. [1]

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Introduction

Protein 53 (p53, Fig. 1) is a well known transcription factor involved in regulating the cell cycle. As a signal which induces apoptosis through Apaf-1-mediated caspase activation, it has been studied by many cancer researchers. [2] B-cell lymphoma-2 (Bcl-2, Fig. 1) can bind to p53. In mammalian cells this binding, being upstream of caspase 9 activation, can inhibit apoptosis. [3] As such, the p53/Bcl-2 interaction is implicated in carcinogenesis.

p53 generally functions as a tetramer whose monomer units each have several domains which can potentially interact with Bcl-2. The goal is quickly to determine which domain is, in fact, responsible for the interaction. As the p53 protein is expressed in *E. coli* with a glutathione S-transferase (GST) tag, this information could be obtained using the GST-pulldown technique. However GST pulldowns would use a large amount of the Bcl-2 protein and require running a gel

whose development is quite time consuming and difficult to quantify.

Instead, the SKi Pro system together with SKi Sensor carboxyl chips are used. Protein interactions are monitored on porous silicon biochips using nano-porous optical interferometry. This technique does not require protein labels in order to determine the yes/no answer required.

Additionally, as the the optical path difference (OPD) is proportional to the amount of protein bound, after it is shown which domain of p53 is responsible for its binding to Bcl-2, the binding affinity may be quantified. By titrating the target p53 domain at several concentrations against the Bcl-2 modified biochips, K_D is calculated. As SKi sensor biochips are available in units of 8 and fit into standard 96-well microtiter plates, the concentration titration is performed in parallel taking about 10 minutes to determine K_D .

This new approach to protein-interaction analysis compares favorably with previous literature values but is automated and requires much less protein and time than other methods.

Method

Bcl-2 protein is purchased from R&D systems (Minneapolis, MN) and used as received. p53 domains are overexpressed in bacteria as GST fusion proteins as shown in Table 1. Each of the domains is interacted separately with the Bcl-2 prepared biochips.

Table 1: Summary of the target proteins, derived from human p53 and used in this study.

Protein Region	Residues
Wild Type	1–393
N-terminal	1–160
Center Region	160–320
C-terminal	320–393

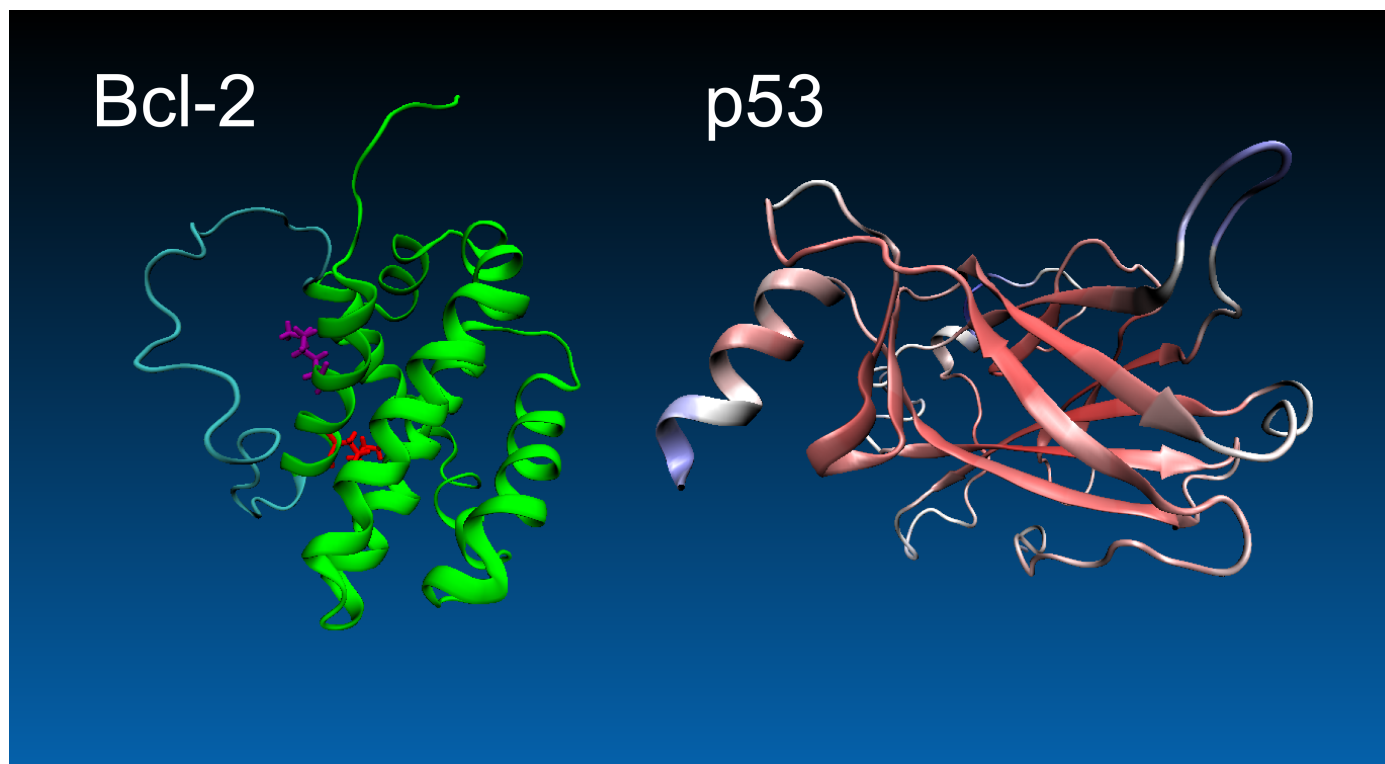


Figure 1: Solution phase NMR structure of the residues 3–207 of the human protein Bcl-2 is shown in **A** [4]. The seven alpha helices are shown in green. The first of these contains the only lysines of the protein K17 in purple and K22 in red. The poorly formed loop between helices 1 and 2 is shown in cyan. The crystal structure for the tetrameric human protein p53 is shown in **B** [5] where residues 96–289 are included. Proteins are rendered from their pdb structures using VMD. [6]

SKi Sensor carboxyl well strips are used for the experiments. The carboxyl groups on the insides of the pores are modified as sulfo-succinimide esters using EDC chemistry during an initial activation step. 100 μL of activation buffer (800 mM EDC, 200 mM sulfo-NHS solution buffered at pH 6.0 with 100 mM MES and 0.9% NaCl) is titrated into each microtiter plate well. The chips are then activated for 15 minutes in this solution and transferred into immobilization buffer (pH 4.5, 20 mM acetate, 1 mM EDTA) and washed twice.

Next, strips are placed into wells, each containing 100 μL of immobilization buffer, and OPD baseline is taken for 10 minutes. The strips are then transferred to a solution of Bcl-2 in immobilization buffer at 1.0 μM in 100 μL of solution per chip. The immobilization proceeds for 45 minutes. Following immobilization, blocking buffer (1,000 mM ethanolamine-HCl, pH 8.0) is applied for 20 minutes to deactivate any unreacted succinimide esters in preparation for specific target binding. Bcl-2 chips as these are used for both the yes/no determination and affinity experiments.

For these binding studies, 100 μL of binding buffer (100 mM PO_4^{3-} , 150 mM NaCl, pH 7.2) is then added

to each of the wells and 10 minutes of baseline OPD monitored. The well strips are then moved into wells containing the proteins used. In the case of the yes/no determination experiments Wild Type p53, N-terminal p53, center region p53, C-terminal p53 and a GST control polypeptide were in each case used at 2 μM concentration in a 100 μL volume.

For the affinity determination of the center region, p53 dose dependent data is taken. 100 μL of protein is measured at concentrations of 0.20, 0.45, 0.67 and 1.0 μM using Bcl-2 modified chips. SKi Report software is then used for method programming, data acquisition and subsequent data analysis.

Results

The preparation of the SKi Sensor carboxyl surface is followed by using SKi Pro to monitor the change in OPD as a function of time. As Bcl-2 covalently binds to the pore walls the value rises by ~ 8 nm as shown in Figure 2. A reference chip, which has no Bcl-2 is shown as well and as expected, remains unchanged throughout the immobilization step.

As proteins bind to the pore walls, they displace the buffer within that pore. Given that proteins have a higher refractive index than the buffer, this binding can easily be monitored by nPOI. In this case the Δ OPD caused by Bcl-2 binding will be irreversible as the binding which occurs is covalent. The succinimide ester chemistry used affects protein conjugation through primary amines. For Bcl-2 that would be either the amino terminus or the two lysine side chains on the first alpha-helix.

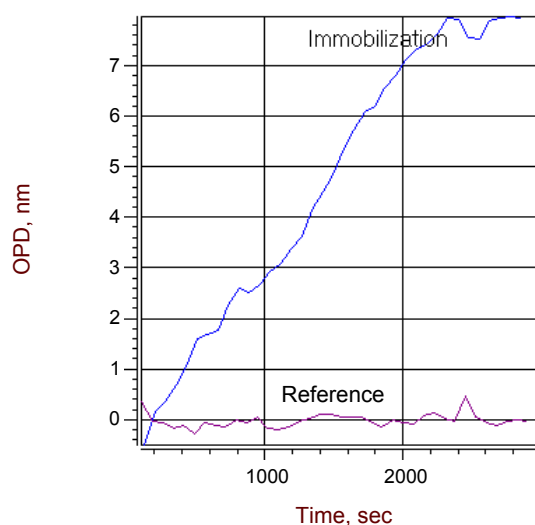


Figure 2: Immobilization of Bcl-2 within the pores of the SKi Sensor chips. The OPD level increases to ~ 7 nm within 2,500 seconds before leveling off. The reference surface—with no protein present—does not show any change.

The known interaction between Bcl-2 and p53 WT was reproduced during the yes/no determination studies shown in Figure 3. Here the $2 \mu\text{M}$ protein solution interacts strongly with the chip showing a Δ OPD of $>2 \mu\text{M}$ in 4 minutes. The center-region p53 protein, also at $>2 \mu\text{M}$ shows even more of a change and does so faster. The N-terminal p53 and C-terminal p53 proteins do not show a change, nor does the GST polypeptide control. The absence of Δ OPD in these cases implies that the signal seen for the wild type and center region are in fact due to specific binding to Bcl-2.

As these concentrations are in each case given with respect to the p53 monomer which has a GST fusion protein attached, it might not be immediately clear why the center region—being smaller—gives a greater signal change. This is likely due to the fact that the binding interaction is not complete within the time shown. The center region, because of its relative size would be expected to interact more quickly and is therefore closer to completion and has a greater Δ OPD.

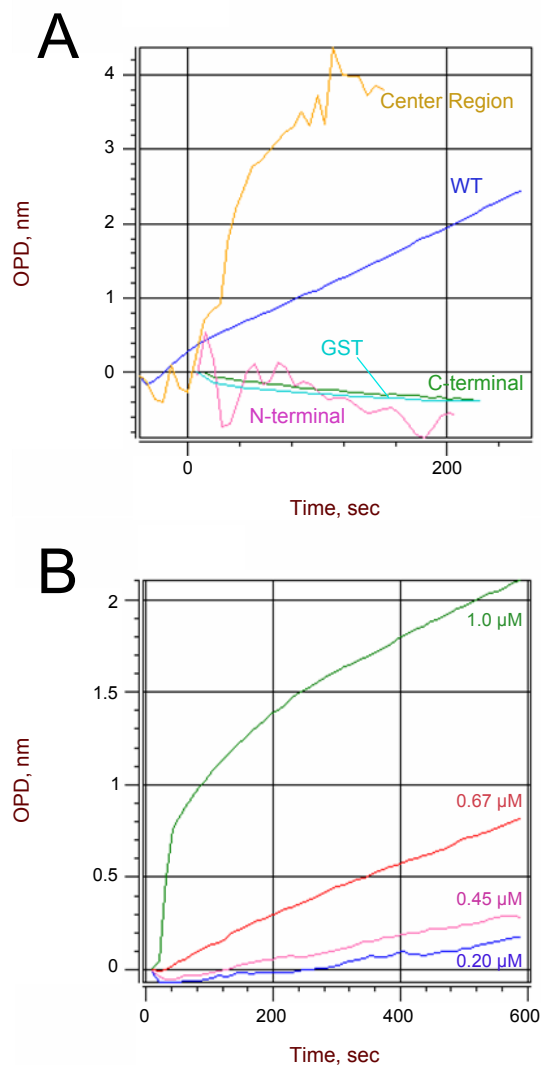


Figure 3: Variants of p53 monitored for yes/no determination on the Bcl-2 surface are shown in A. All proteins shown in Table 1 are interacted to see if binding occurs. The wild type and center region proteins interact whereas N-terminal, C-terminal and GST control do not. After identifying the center region as responsible for the binding, this was titrated against Bcl-2 with the results shown in B. The Δ OPD shift between 500–600 seconds is then averaged, plotted and fit to determine a K_D of 670 nM.

As it is established that the center-region of p53, as opposed to the N-terminal and C-terminal regions, is responsible for the interaction between Bcl-2 and p53, this region's affinity is characterized by dose response in Figure 3B. The Δ OPD ranges from 0.2 nm in the case of the $0.20 \mu\text{M}$ sample to 2.1 nm for the $1.0 \mu\text{M}$ case in 10 minutes. The kinetics are visibly faster in the case of the higher concentration but explicit kinetics models were not used in the analysis.

Discussion

nPOI allows a rapid determination of both the region of p53 responsible for the Bcl-2 interaction and the magnitude of the interaction's affinity. The label free microtiter plate method allows the researcher to study protein-protein interactions quickly after recombinant protein purification. Furthermore, the unique chemistry of the SKi Sensor biochips reduces non-specific binding to low enough levels, that GST fusion proteins may be used as purified.

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