An NMR-Based Antagonist Induced Dissociation Assay for Targeting the Ligand–Protein and Protein–Protein Interactions in Competition Binding Experiments

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We present an NMR-based antagonist induced dissociation assay (AIDA) for validation of inhibitor action on protein–protein interactions. As opposed to many standard NMR methods, AIDA directly validates the inhibitor potency in an in vitro NMR competition binding experiment. AIDA requires a large protein fragment (larger than 30 kDa) to bind to a small reporter protein (less than 20 kDa). We show here that a small fragment of a protein fused to glutathione S-transferase (GST) can effectively substitute the large protein component. We successfully used a GST-tagged N-terminal 73-residue p53 domain for binding studies with the human MDM2 protein. Other interactions we studied involved complexes of CDK2, cyclin A, p27, and the retinoblastoma protein. All these proteins play a key role in the cell division cycle, are associated with tumorigenesis, and are thus the subject of anticancer therapy strategies.

Introduction

NMR-based methods for screening for drug candidate compounds that bind protein targets can be divided into two main classes. In the first class, NMR signals of low molecular weight compounds are monitored by utilizing NOE effects or differential relaxation rates of their free and bound states. The second approach focuses on the changes in the ligand-induced NMR chemical shifts of the protein. The latter class of the methods requires larger amounts of isotopically labeled proteins but can provide more detailed information on the ligand–protein interaction by showing where compounds bind on their target proteins. For proteins of small size (i.e., less than 20 kDa) the most popular protocol has been based on the use of chemical shift perturbations in 2D 1H–15N HSQC spectra of uniformly 15N-labeled proteins. The second approach focuses on the changes in the ligand-induced NMR chemical shifts of the protein. The latter class of the methods requires larger amounts of isotopically labeled proteins but can provide more detailed information on the ligand–protein interaction by showing where compounds bind on their target proteins. For proteins of small size (i.e., less than 20 kDa) the most popular protocol has been based on the use of chemical shift perturbations in 2D 1H–15N HSQC spectra of uniformly 15N-labeled proteins. For larger proteins, when 15N labeling is not sufficient to resolve spectral overlap in 2D 1H–15N spectra, selective amino acid labeling or protein perdeuteration may be necessary and TROSY type of experiments are advantageous over “traditional” HSQCs.

In principle, the assignment of NMR resonances is not required if the only purpose of the NMR experiment is to detect the binding of ligands to target proteins. To monitor a specific binding site on a protein, active site residues must be identified, for example, by experiments with known ligands, or for a more detailed structural interpretation, the interface can be determined on the basis of a known 3D structure and NMR assignment procedures.

The NMR screening techniques concentrated so far on monitoring binary ligand–protein interactions. For protein–protein interactions, the ultimate goal of an antagonist compound discovery is to find a lead compound that inhibits or dissociates these interactions. Binding to a target protein does not directly imply this desired result. On the other hand, a compound that does not bind to one component of the complex can still suppress complex formation by interacting with the second protein; thus, screening for both targets would be necessary. We have recently developed an in vitro NMR method for studying the effect of antagonists on protein–protein interactions. The method, named AIDA NMR (for antagonist induced dissociation assay by NMR), requires the protein complex of interest to be formed between proteins with considerably varying molecular weights, one of which is labeled and should give a good quality S/N NMR spectrum and would serve as an NMR reporter protein. Formation of the complex should result in higher transverse relaxation rates R2 of the reporter protein, which causes a decrease in its NMR intensities to a point that they may completely disappear. This clear indication of the interaction can be easily detected and this part of the experiment must be performed only once, at the time of the system and conditions selection.

The spectrum of the protein–protein complex changes dramatically if an inhibitor that breaks the complex is added. Two outcomes are possible in the case of the complex dissociation: either the reporter spectrum is recovered unchanged, when the inhibitor binds to the larger protein, or the recovered spectrum displays modifications caused by the inhibitor binding. It is noteworthy to point out that the dissociation is detected irrespective of the protein the compound acts on; thus, there are two targets checked simultaneously and the selection of the right binding site and the inhibition strength is obtained in principle without any prior knowledge of these parameters. Our approach therefore targets protein–protein interactions and not a single protein. A schematic representation of our method for a two-protein complex is shown in Figure 1.

AIDA also provides information whether its action is through denaturation, precipitation, or release of the protein in its functional folded state. We have checked this method on three lead compounds that have recently been reported to inhibit the p53–MDM2 interaction; only one of them turned out to be a potent inhibitor of this interaction. Disrupting the MDM2–p53 interaction (and more general also MDMX–p53) would rescue the impaired p53 function, and thus...
inhibitors of the p53–MDM2 interaction offer a new avenue for cancer therapy. To monitor the influence of potential antagonists on the p53–MDM2 interaction, we used a 15N-labeled 118 amino acid N-terminal domain of MDM2 and a 312-residue N-terminal fragment of p53. In the present report, we describe our NMR experiments for the GST-tagged N-terminal 73-residue p53 domain, which replaces the 1–312 residue p53 fragment. The possibility to work with the GST-tagged domains would make our NMR protocol universal, as these are protein constructs that are routinely obtained in molecular biology preparations. We further tested our protocol on pure multiprotein interactions, which involved competition of two small protein fragments, the C-terminal domain of the retinoblastoma protein, and the N-terminal domain of p27, for binding to a large complex of CDK2/cyclinA. All these proteins play a fundamental role in the regulation of the human cell division cycle.

Results

In the first experiment the isotopically 15N enriched fragment of MDM2 (residues 1–118) (Figure 2a) was titrated against the unlabeled GST-p53 (residues 1–73). Complex formation was observed by the disappearance of most of the MDM2 peaks, as seen in Figure 2b. The leftover peaks originate from flexible residues of MDM2 in the complex and are located in the spectrum at the “central 8.3 ppm NH amide” region, diagnostic for unstructured residues, plus side chains at 7 and 7.5 ppm. We have used nutlin-3 as a positive control for our NMR assay. Nutlin-3 is the most potent inhibitor out of the three nutlins, a class of cis-imidazoline compounds recently reported as inhibitors of the p53–MDM2 interaction. Addition of nutlin-3 to the GST-p53 domain, which replaces the 1–312 residue p53 fragment. The possibility to work with the GST-tagged domains would make our NMR protocol universal, as these are protein constructs that are routinely obtained in molecular biology preparations. We further tested our protocol on pure multiprotein interactions, which involved competition of two small protein fragments, the C-terminal domain of the retinoblastoma protein, and the N-terminal domain of p27, for binding to a large complex of CDK2/cyclinA. All these proteins play a fundamental role in the regulation of the human cell division cycle.

Figure 1. Schematic representation of the AIDA method for studying the effect of an antagonist on the interaction between two proteins.

Figure 2. Spectra of the 15N uniformly labeled MDM2. (a) 1H–15N HSQC spectrum of 15N-MDM2. (b) 1H–15N HSQC spectrum of 15N-MDM2 complexed with p53. Most of the cross-peaks disappear from the reference MDM2 spectrum, indicating complex formation. (c) 1H–15N HSQC spectrum of MDM2 in complex with nutlin-3. Some cross-peaks are shifted (compared to Figure 2a) due to binding of nutlin to MDM2.

Discussion

The benefit of our method is the direct observation of the action of an inhibitor or a protein without prior knowledge of structure/assignment information. The method covers also large
protein complexes and protein/polypeptide inhibitors, encountering the limit rather in sample concentration (or the spectrometer sensitivity) than in the multiprotein complex size, provided that one component of the complex alone gives a good spectrum. A dramatic change in the spectrum in the case of ligand binding offers comfortable and reliable automation opportunities.

In the current study, we have used a 118 amino acid N-terminal domain of MDM2 and the GST fusion N-terminal 73-residue fragment containing the transactivation domain of p53 (a schematic of the full-length proteins is shown in Figure 5). The binary complex has a total molecular weight of 50 kDa and a $K_D$ of ca. 0.7 $\mu$M. We show experimentally in this report that GST-p53(1–73) can substitute for p53(1–312) in our assay. Although GST is known to form dimers, GST-p53 eluted as a monomer in gel filtration experiments.

Since our method is a competition assay, it is essential to understand how strong a binder the ligand must be in order to dissociate the protein–protein interaction. In principle, the effect of a ligand will depend on the strength of the protein–protein interaction, concentrations of the ligand and protein, and the inhibitor–protein affinity. The sensitivity of the NMR spectrum is also important, as we have to know how much of the protein–protein complex must be dissociated in order to detect partial recovery of the reporter protein in the NMR spectra. We have used ca. 0.1 mM concentrations for protein complexes, and in our practice we found that 30% of the dissociated MDM2 is required for a clear-cut detection by $^{15}$N HSQCs; thus, we will use this value in further discussion. An exact analytical solution to the competition problem was published by Wang and was used for applications in the isothermal titration calorimetry (ITC) by Sigurskjold. Below we present considerations for the conditions specific to our NMR experiments.

First, we have to know how much of a free reporter protein is present in the sample prior to the ligand addition. The equilibrium concentrations of the reporter, the binding protein, and the protein–reporter complex are denoted $[R]$, $[P]$, and $[PR]$, respectively. $K_D$ is the dissociation constant of the complex, and $[R]_0$ and $[P]_0$ are the total concentrations of the reporter and protein ([R]$_0$ = [R] + [PR], [P]$_0$ = [P] + [PR]). The ratio of the free reporter concentration to the total reporter concentration $r = [R]/[R]_0$ is

$$r = \frac{1}{2} \frac{\sqrt{(k+\alpha-1)^2 + 4k}}{k + \alpha - \sqrt{(k+\alpha-1)^2 + 4k}}$$

Figure 3. Spectra of the $^{15}$N uniformly labeled pRb-C. (a) effects of cyclinA/CDK2 addition: red, initial spectrum; green, ca. 50% complexed. Double peaks indicate slow exchange. (b) Fully complexed pRb-C (blue) and after addition of p27 initial spectrum is restored (pink).

Figure 4. (a) A $^{15}$N-HSQC spectrum of $^{15}$N-labeled p27 (a) and after addition of cyclinA/CDK2 complex (b). Most of the peaks disappeared. Addition of the substance that dissociates the complex should restore the spectrum. The system seems to be well-suited for the inhibitor research.

Figure 5. Superposition of HSQC spectra of $^{15}$N-labeled p53 (residues 1–312, red) and GST-p53 (residues 1–73, blue).
1), where $k = K_D^C/[R]_0$ and $\alpha = [P]_0/[R]_0$. It can be found by solving a quadratic equation. It is illustrated in Figure 6a.

Typically, the NMR sample concentration is higher than or close to 0.1 mM, while the $K_D$ is below 10 nM; thus, $k < 0.1$. For $\alpha = 1.0$, one obtains $r = 0.27$, which is near to the 30% detection threshold but decreases with higher binding strengths and sample concentrations. Figure 6a shows how the free reporter concentration drops for smaller $k$. One can also consider using an excess of the binding protein, thus increasing parameter $\alpha$ in the above equation.

The second question is how much of an inhibitor should be added to cause a detectable effect. Two cases can be considered. In the first case, when the inhibitor binds to the reporter there are two types of molecules that potentially can be detected (they have molecular weight smaller than 30 kDa): an inhibitor reporter complex and the free reporter protein. For simplicity, we assume that free reporter is undetectable in this case. In the second case, when inhibitor binds to the protein interacting with the reporter, the only detectable molecule is free reporter. For further discussion we found it convenient to express the total ligand concentration $[I]_0 = [I] + [RI]$, as a parameter, $\beta = [I]_0/[R]_0$. One can then plot a curve of $\beta$ necessary to release a given percentage (30%) of the reporter (or inhibitor reporter complex) for different $K_D^C/K_D^R$ ratio (Figure 6b,c). The shape of this curve depends on $K_D^C/[R]_0$; however, for NMR conditions, where this value is usually below 0.1, it is almost universal (Figure 6d).

The first conclusion is that the concentration of an uncomplexed reporter protein often can be neglected. The second is that if the large binding component protein or the reporter protein have considerably higher affinity to the ligand than to the other binding partner, it will bind mostly to the added inhibitor and dissociation of the reporter protein complex will be detected at total concentration of an inhibitor similar to the total concentration of the protein—reporter complex component which is replaced by the inhibitor. If, on the other hand, the affinity to the ligand is 10 times, or more, weaker than the binding between the two proteins, then the complex can be dissociated at detectable levels only when a huge excess of the ligand is used. Note that a ligand that binds to the target protein "tightly", e.g. 0.5 μM, might require an excess of a ligand exceeding its solubility limit, if the $K_D$ of the complex is, for example, below 10 nM, and thus would be deemed to be ineffective. Our experimental protocol can be still adapted for the case of an extremely tight binding or an irreversible chemical reaction. In such cases, effective inhibition requires the presence of the tested inhibitor compound prior to the complex formation (Figure 7). In this variant, the chemical shift changes caused by the compound in the reporter spectrum can be measured on the same sample. Regardless of which protocol was used for

Figure 6. Equilibrium concentrations of selected compounds. (a) Binary mixture, free reporter concentration versus different $K_D$ to reporter concentration ratio, for 1.0, 1.25, and 1.5 molar ratio of the complex substrates. (b) Ternary mixture. Amount of inhibitor (in [R]_0 units) necessary to bind 30%, 50%, or 70% of reporter molecules for different binding strength ratio, and 1.0 or 1.5 molar ratio of complex substrates, found numerically at $K_D^C/[R]_0 = 0.001$. (c) Amount of inhibitor (in [R]_0 units) necessary to reach 30%, 50%, or 70% of free reporter molecules for different binding strength ratio, found numerically at 1.0 and 1.5 molar ratio of complex substrates and $K_D^C/[R]_0 = 0.001$. (d) Illustration on how the curve from part b depends on $K_D^C/[R]_0$.

Figure 7. An AIDA protocol for an irreversible reaction.
the complex formation, a known strong inhibitor that dissociates
the complex can be used for a positive control if the tested
compound did not cause detectable effects (see Krajewski et al.18). Our method tests this property directly, while the “SAR
by NMR” approach requires comparison of the dissociation
constants of the protein complex and ligand—target interaction.
Such information can be considerably difficult to obtain at
required precision in the case of tight binding, whereas AIDA-
NMR can provide hints about how much the affinity must be
improved.

We would like to note that our method is neither in
competition with the traditional SAR by NMR7,10–12 nor can it
be considered as a variant of methods described in the
Introduction. Although the common final goal of all these
methods is to find a potential inhibitor, they use different
paradigms to achieve it. The AIDA-NMR and SAR by NMR
methods provide different types of information, and being
suitable for different situations, they well supplement each other.
The SAR by NMR approach cannot be used when only large
fragments of proteins are available, which may be the case at
the beginning of the characterization of protein—protein binding.
Also, many important minimal domains of proteins are about
300 amino acids in length. For large proteins of ca. 30 kDa,
the HSQC spectra are normally too crowded to be of practical
use in these types of experiments. Such systems are often perfect
for the method we present. AIDA-NMR, on the other hand,
does not provide directly structural information on how to design
or improve the inhibitor.

Increased $T_2$ relaxation rates in our NMR assays were
achieved through the formation of large molecular weight
complexes. Several other methods may be used to achieve a
similar effect, like, for example, complexing with paramagnetic
probes. The method can possibly be used for monitoring factors
necessary for a polymerization or depolymerization reaction.
The requirement is that one of the complex members gives a
good NMR spectrum when free in solution and that its transverse
relaxation rates would be distinctly higher after the reaction.

**Methods**

**Protein Expression and Purification.** The recombinant human
MDM2 protein was obtained from the *Escherichia coli* BL21(DE3)
RIL expression system and contains the first 118 N-terminal
residues of human MDM2 cloned in a pET46 Ek/LIC vector
(Novagen). The inclusion bodies were washed with the PBS buffer
containing 0.05% Triton X-100 with subsequent centrifugation at
12 000g and solubilized with 6 M guanidine hydrochloride in 100
mM Tris-HCl, pH 8.0, including 1 mM EDTA and 10 mM DTT.
After lowering the pH to 3–4, the protein was dialyzed overnight
at 4 °C, against 4 M guanidine hydrochloride, pH 3.5, including
10 mM DTT. For renaturation, the protein was diluted (1:50–1:
100) into 10 mM Tris-HCl, pH 7.0, including 1 mM EDTA and
10 mM DTT by adding the protein in several pulses to the refolding
buffer. Refolding was performed overnight at room temperature.
Ammonium sulfate was added to a final concentration of 1.5 M
and after ca. 1 h butyl sepharose 4 Fast Flow (Pharmacia, FRG)
was added to the refolded human MDM2. We used a glass-frit
funnel as a filter to collect MDM2—butylsepharose. The protein
was eluted with 0.1 M Tris-HCl, pH 7.2, supplied with 5 mM DTT.
Finally, all fractions containing MDM2 were pooled, concentrated,
and applied to a HiLoad Superdex 75 pg gel filtration column
(Pharmacia, FRG). The running buffer contained 50 mM KH2PO4,
50 mM Na2HPO4, pH 7.4, 150 mM NaCl, 5 mM DTT. All fractions
with the monomeric human MDM2 were pooled and concentrated
for NMR spectroscopy.

The recombinant human GST-p53 protein (residues 1–73) was
overexpressed at 37 °C in *E. coli* BL21 (DE3) using a pGEX vector.
The protein was purified using a GST Sepharose Fast Flow column
(Amersham). The recombinant p27 (residues 1–96) was overexpressed
at 37 °C in *E. coli* BL21 (DE3) using a PET28 vector. The protein
was purified under native condition using Ni-NTA Agarose
(Qiagen). The C-terminal construct of pRB residues (residues 801–
890) was overexpressed in *E. coli* BL21 (DE3) overnight at 20 °C
using a PET 46 vector and purified under native condition using
Ni-NTA Agarose (Qiagen). The final purification of all proteins
was done with a HiLoad 16/60 Superdex75 gel filtration column
(Amersham). CDK2 and cyclinA were expressed separately in *E.
coli* BL21 (DE3) at 20 °C using a PET 46 vector. They were first purified
under native condition using Ni-NTA Agarose (Qiagen) and
second a HiLoad 16/60 Superdex75 gel filtration column
(Amersham). The two purified proteins were mixed in equimolar
concentrations, and the complex was separated from the monomers
by a second gel filtration. The uniformly $^{15}$N-enriched protein
samples were prepared by growing the bacteria in minimal medium
containing $^{15}$N-labeled NH4Cl.

**NMR Spectroscopy.** All NMR spectra were acquired on a
Bruker DRX 600 MHz spectrometer equipped with a cryoprobe
except the pRb-C titration experiment, which was done on the DRX
500 MHz with a standard triple resonance probe. Typically, NMR
samples contained up to 0.2 mM (0.5 mM at the 500 MHz
spectrometer) of protein in 50 mM KH2PO4, 50 mMNa2HPO4, 150
mM NaCl, pH 7.4, 5 mM DTT, 0.02% NaN3, and protease
inhibitors or 40 mM HEPES, 200 mM NaCl, 5 mM DTT for the
experiments with CDK2/cyclinA. The MDM2/p53 system was
measured at 300 K, while titration of pRb-C with CDK2/cyclin
was carried out at 283 K. For the $^{1}H-^{15}N$ HSQC spectrum,8 a
total of 2048 complex points in $t_1$ and 128 $t_2$ increments were
acquired. Water suppression was carried out using the WATER-
GATE 5 sequence. NMR data were processed using the Bruker
program Xwin-NMR version 3.5. Titration experiments were
performed using a series of $^{1}H-^{15}N$ HSQC of labeled p53 or
MDM2 along with the unlabeled partner. By monitoring the 1D
proton spectra, care was taken to prevent overtitration of the
unlabeled sample.

**Supporting Information Available:** An analytical solution for
a chemical equilibrium in the case of competitive binding. This
material is available free of charge via the Internet at http://
pubs.acs.org.

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Antagonism of Protein—Protein Interactions


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