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NMR Probing of Protein−Protein Interactions Using Reporter Ligands and Affinity Tags

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In a living organism, proteins are not isolated entities but interact in a spatially and temporally controlled manner with their respective binding partners.[1,2] A wide variety of methods have thus been developed to study such interactions.[3−7] Although an enormous amount of data has been acquired in the past (e.g., comprehensive analysis of protein complexes in entire genomes), the importance of verifying their relevance has been emphasized.[8−10] For in vitro analysis of direct protein interactions, a large variety of methods exists, including affinity chromatography, electrophoresis, fluorescence spectroscopy, and calorimetry. Recently, an NMR method employing 1D ^{1}H or ^{13}C-edited ^{1}H NMR was proposed for detecting protein−protein interactions in solution.[11] Although no protein modification is required, applications of this method are limited, however, by the fact that the interacting proteins are directly observed.

Here we propose a different strategy. In our approach, one protein binding partner is fused to a ligand binding domain, and protein−protein interaction is monitored via changes in the NMR relaxation of a medium-affinity ligand which reversibly binds to the ligand binding domain. NMR spectroscopy is well-established as a powerful method for studying interactions between a ligand and a macromolecular target, particularly for screening applications in the pharmaceutical industry.[12] Specifically, ligand-observed screening is very attractive because it is not limited by the size of the protein, does not require isotope-labeling, and is usually employed using low protein concentrations. It requires, however, the prevalence of medium-to-fast exchange regime (μM K_D values), since only the unbound form of the ligand is observed due to the large molar excess of the ligand. The change in the spectral parameter of the free ligand protons (e.g., selective T_1, T_2, or intermolecular water NOE efficiency) depends on the affinity constant, the concentrations of ligand and protein, and the molecular weight of the protein−ligand complex.

While in conventional NMR screening applications the emphasis is in finding ligands with an affinity to the target of interest, here the changes in NMR parameters are used to probe changes in the molecular composition of the ternary protein−ligand complex. An outline of the method is given in Figure 1. The relaxation time of a particular ligand proton is changed upon binding to the ligand binding domain. If the fusion protein subsequently binds to another protein (via the protein which is attached to the ligand binding domain), the resulting relaxation of the ligand will be determined by the molar ratios and the molecular weights of all components in the system: free ligand, binary fusion protein ligand complex, and the ternary protein/protein/ligand complex. Thus, the primary advantage of this novel reporter ligand method for protein−protein interaction monitoring is the fact that the reporter ligand is used for NMR observation and that the proteins are present only at very low concentration.

As an application, we show the ligand-observed interaction of the oncogenic transcription factor v-Myc with its authentic protein

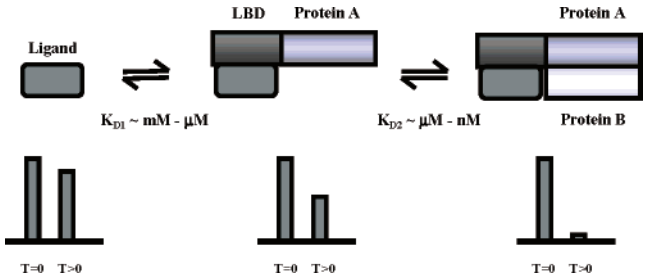

Figure 1. Outline of the NMR protein−protein interaction reporter system. A ligand binding domain (LBD) with medium affinity (K_{D1}) to a small molecular weight compound is first attached to the target protein A using standard molecular biology techniques. The physical interaction between the protein A and a potential protein binding partner B (K_{D2}) is probed via changes in the relaxation behavior of the small molecular weight ligand reversibly bound to the ligand-binding domain (differential intensity changes during the relaxation period T). The change in ligand relaxation rate results from the increase of the molecular weight upon binding protein B and is determined by the effective molecular weight and molar ratio of the ternary protein−ligand complex.

binding partner Max. v-Myc was shown to tightly interact with Max, and the heterodimeric protein complex binds to DNA and activates transcription.[13] Earlier work revealed that apo v-Myc exists in solution as a partially folded protein displaying two helical segments (e.g., the basic, DNA-binding region and the C-terminal leucine zipper region).[13] The relative orientation of the two helical domains is undetermined in the free form. However, upon v-Myc−Max heterodimerization the two proteins form a stable four helix bundle structure with a well-defined leucine zipper and hydrophobic core region, respectively. The SH2 domain from PLCγ1 was chosen as the ligand binding domain. SH2 domains bind phosphotyrosine-containing peptides with high specifity. However, to ensure fast-to-medium exchange conditions we chose phenyl phosphate as the reporter ligand. The dissociation constant K_D of the phenylphoshate PLCγ1(SH2) complex was determined to be 1mM. v-Myc was fused to the SH2 domain using standard molecular biology methodology. In brief, to generate a suitable SH2 fragment a polymerase chain reaction was performed using pET11d-PLCγ1CSH2 as a template and GGAGATATCATATGGGTTCACCGGGAATTC-AC and ATCCACCATGGATGAATTCTCCTCGTTGATG as 5′ and 3′ primers, respectively, to introduce a *Nde*I and a *Nco*I restriction site for the following digestion.[14] A *v-myc* fragment was obtained by cleaving pET3d−p15myc with *Nco*I and *BamH*I. Both fragments were cloned into a pET42 vector (Novagen) predigested with *Nde*I and *Bam*HI.[13] The resulting plasmid was transformed into *Escherichia coli* Rosetta pLysS cells. The proteins were expressed and purified as described previously.[13,14]

The binding of phenyl phosphate to the SH2 domain was monitored by selective T_1 measurements of the aromatic proton in meta position to the phosphate group. Figure 2 shows experimental results obtained with selective T_1 measurements. Upon binding to the protein Max the molecular weight of the protein−ligand complex was

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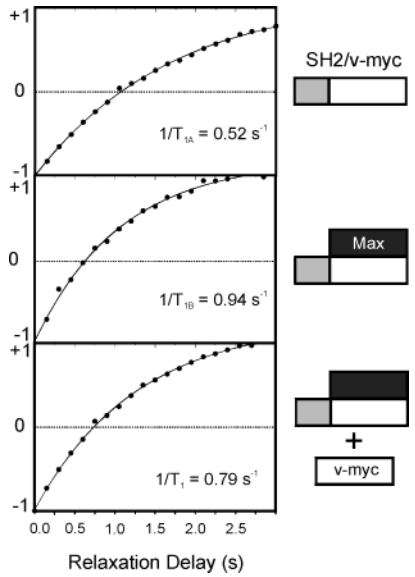

Figure 2. Experimental demonstration of the ligand-based NMR protein−protein interaction detection scheme. The oncogenic transcription factor v-Myc is fused to the SH2 domain (gray box), and the interaction with its authentic protein binding partner Max is studied in vitro. Selective T_1 recovery curves of the meta proton of phenyl phosphate reversibly bound to (top) tagged v-Myc, (middle) tagged v-Myc in complex with Max, and (bottom) a 1:1 mixture of tagged v-Myc and untagged v-Myc in complex with Max. The experimental selective relaxation rates ($R_1 = 1/T_1$) are given. Selective inversion and excitation were achieved using IBURP (22 ms, $\gamma B_1 = 250$ Hz) and EBURP (20 ms, $\gamma B_1 = 215$ Hz) pulses, respectively.[15] The concentrations of the ligand and the proteins were as follows. Phenyl phosphate: 500 μM; SH2/v-Myc: 100 μM; Max: 100 μM; v-Myc: 100 μM. The experiments were performed on a Varian Unity Inova 800 MHz spectrometer. The inversion recovery spectra were acquired with 64 transients and using a relaxation delay of 5 s. Sample conditions: pH = 7.0, $T = 25$ °C.

increased, which led to an increase of the selective relaxation rate $R_1 = 1/T_1$. The change in R_1 was determined by the protein concentrations, the protein−protein affinity (K_D of protein−protein complex), and the molecular weight of the resulting protein−protein complex. The moderate but significant change in the selective relaxation rate (v-Myc: 0.52 s^{-1}; v-Myc−Max: 0.94 s^{-1}) was due to the weak affinity of phenyl phosphate to the SH2 domain of PLCγ1. Phenyl phosphate was chosen to provide a proof of concept. For improved sensitivity and reduced amount of protein material, of course, higher affinity SH2 ligands are preferable. We are currently investigating substituted phenyl phosphate molecules with improved affinities to the SH2 domain of PLCγ1. As a next step, we tested the possibility to perform and quantify competition binding experiments (e.g., replacement of tagged protein in the protein−protein complex through untagged wild-type protein and/or mutant proteins). Competition binding experiments were performed with equimolar amounts of untagged v-Myc, which replaced tagged v-Myc (fused to SH2) in the v-Myc−Max protein complex (Figure 2). The addition of untagged v-Myc released tagged SH2/v-Myc from the protein complex and thus reduced the average molecular weight of the ligand binding domain. The observed change in the selective relaxation rate ($R_1 = 0.72$ s^{-1}) was used to calculate the molar ratios of the bound and the free form of SH2/v-Myc. The fraction of the bound form of SH2/v-Myc was calculated to be 0.65, which was slightly larger than expected (0.5). This was due to a stabilization of the tagged SH2/v-Myc−Max compared to the untagged v-Myc−Max protein complex and was independently corroborated by CD spectroscopic melting curves (data not shown).

In conclusion, we have shown that protein−protein interactions can be efficiently detected using this novel NMR reporter system. The primary advantage comes from the fact that due to the indirect detection only minute amounts of protein material and no isotope labeling are required. A substantial reduction of protein material can be expected by exploiting the large CSA contribution of the ^{19}F nucleus for ligand-based detection of protein−ligand interactions.[16,17] We have also successfully demonstrated that competition experiments are feasible and can be used, for example, for protein−protein interaction site mapping via Ala-mutational screening. For quantitative analysis of protein−protein interactions, however, one has to consider the possibility that the protein binding partner directly interacts with the reporter ligand and/or protein interaction may be altered by the presence of the ligand binding domain, and careful control experiments are thus indispensable. Finally, we anticipate that its ease of implementation and the high-throughput capability will make the method an attractive tool for protein interaction inhibitor screening.

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