Spin Label Enhanced NMR Screening

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One of the most powerful features of biomolecular NMR spectroscopy is its ability to study molecular interactions.[1] An important application can be found in drug discovery research, where NMR has recently gained considerable attention since it can detect even weak binding interactions between a protein target and a ligand. Consequently, NMR screening has been established as an alternative binding assay for lead generation and lead validation.[2] While the advantages of NMR screening are robustness and sensitivity for weak interactions with dissociation constants in the millimolar range, its disadvantage is the requirement for large amounts of concentrated protein. This limits its applicability to proteins that express well and are soluble up to high micromolar concentrations. We recently showed that for second-site NMR screening, where a second ligand is sought which binds to the target protein simultaneously to a known ligand, this disadvantage can be overcome by spin-labeling the first ligand.[3] The spin label, a paramagnetic moiety with an unpaired electron, significantly reduces protein demands due to the dramatic relaxation effects it exerts on neighboring protons.[4] Here we show that the advantageous properties of the spin label can be utilized also for primary NMR screening. By doing so, we reduce the protein requirement by 1 to 2 orders of magnitude, and alleviate the necessity of high ligand solubility.

Unpaired electrons, the essential feature of spin labels such as 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO) or 2,2,5,5-tetramethylpyrroline-1-oxyl, drastically increase the relaxation of neighboring protons due to the high gyromagnetic ratio, which is 658 times larger than that of a proton. In practice, the resonances of protons that are within a distance of 15−20 Å from the paramagnetic center are significantly broadened, and their signals are weakened or quenched when a short spin lock period precedes detection of the NMR signal.[3,5] Since the average distance between molecules is much larger than 15−20 Å in dilute solutions (1−100 μmol/L), intermolecular relaxation enhancement is negligible unless molecular interactions establish binding affinity between two molecules that result in a reduced average distance between them. In the case of second-site screening, it was shown that only the presence of a protein target, to which both ligands had weak affinity, conveyed the relaxation effects from the spin label to the second ligand.[3]

If this method is to be used for primary NMR screening, the protein target itself has to be spin labeled. This is readily achieved at certain amino acid side chains by applying established techniques of protein modification.[4,6] Any ligand that comes close to the spin label, due to its affinity to the protein target, experiences paramagnetic relaxation enhancement caused by the spin label, so that its resonances are broadened and weakened (Figure 1).

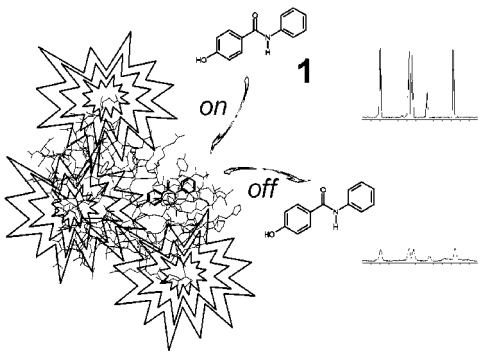

Figure 1. Principle of the SLAPSTIC method. Small organic compounds in solution have typically sharp NMR resonances. When a compound is bound to the spin-labeled protein target, however, it experiences drastic relaxation effects by the spin label. Upon dissociation, this compound can easily be identified by its broadened and weakened resonances.

We demonstrate the power of the SLAPSTIC method (*s*pin *l*abels *a*ttached to *p*rotein *s*ide chains as a *t*ool to identify *i*nteracting *c*ompounds) by applying it to primary NMR screening to identify ligands for the FK506 binding protein, FKBP.[7] FKBP has several lysine residues within a radius of 12−15 Å from the binding site of the pipecolinic acid moiety. We therefore chose to spin label lysine residues, which is easily achieved within a few hours by using well-established methods.[8] To demonstrate the technique, we prepared a mixture of *p*-hydroxybenzanilid **1**, which binds to FKBP with a dissociation constant of 1.1 mM,[9] and four nonbinding aromatic compounds. Figure 2 shows proton spinlock spectra of this mixture, with a short (10 ms, upper row) and long (200 ms, lower row) spinlock period. In proton spinlock spectra, ligands to a target protein are identified by a strongly decreased signal intensity after a long spinlock period. Proton spinlock spectra were recorded in the absence of target protein (left), in the presence of 60 μM FKBP (middle), and in the presence of 20 μM spin-labeled FKBP (right). Clearly, the affinity of *p*-hydroxybenzanilid to FKBP is most easily detected by using spin-labeled FKBP, since this leads to a complete quenching of

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(6) Lundblad, R. L. *Techniques in protein modification*; CRC Press: Boca Raton, 1995. A variety of protein side chains can be chemically modified and spin labeled, including lysine, cysteine, histidine, glutamate, aspartate, tyrosin, and methionine. The residue type of choice for labeling can be selected for each individual case by inspection of the binding site.

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(8) All NH_2 groups in FKBP (lysine side chains and N-terminal NH_2) were substrates for spin-label modification by reaction with 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylate *N*-hydroxysuccinimide ester (Toronto Research Chemicals, O87400). Spin label reagent (66 μL: 10 mg dissolved in 100 μL DMSO) were added to 9 mL of FKBP solution (1.6 mg/mL in 50 mM sodium phosphate, pH 7.5). The solution was mixed on a rotatory shaker for 2 h at room temperature and finally filtered through 0.45 μm and gel filtered on Superdex G25 fine equilibrated and run in 50 mM NaPi, pH 7.5, to remove excess reagents. The average number of spin labels per FKBP molecule as determined by HPLC-MS was 3−4. In the course of our work, we realized that spin-labeled FKBP lost some of its quenching activity over a time course of several months at 4 °C, presumably due to reduction of the spin label. We therefore recommend to store the spin-labeled protein in a cool place, to degas the solution, and to add EDTA.

(9) This K_d was determined for the binary complex between FKBP and compound **1**. The K_d of **1** in the presence of saturating amounts of a pipecolinic acid derivative has previously been determined to be 0.6 mM,[2a] suggesting cooperativity in binding. In our titration, a 1 M stock concentration of **1** was employed, to reduce artifacts due to DMSO binding.[10]

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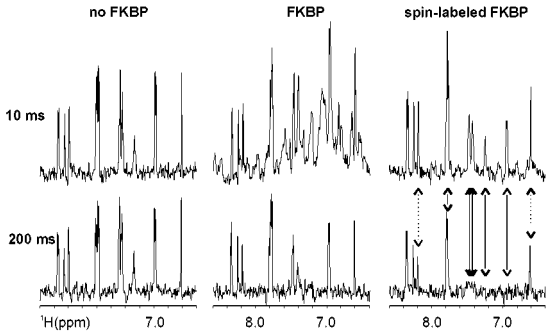

Figure 2. T1ρ relaxation experiments of a mixture of **1**, **2**, and four nonbinding compounds, with spin lock periods of 10 (upper row) and 200 ms (lower row). The compound concentration was 50 μM, and protein concentrations were 0 (left), 60 μM nonmodified FKBP (middle), and 20 μM spin-labeled FKBP (right). The measuring time for each spectrum was 90 s. Signal quenching of **1** (solid arrows) is most easily detected with spin-labeled FKBP (right), which identifies **1** as a ligand for FKBP. Dotted arrows indicate resonances of methyl-4-methoxythiophene-3-carboxylate, which binds to FKBP very weakly (K_d = (9 ± 2) mM).[10] There are two signals at 7.8 ppm, one from compound **1**, and one from a nonbinding compound.

its resonances. Binding affinity of **1** to FKBP can also be detected by using nonmodified FKBP, but in this case the relaxation effects are much smaller. Relaxation effects similar to the ones caused by 60 μM nonmodified FKBP are achieved by using 1 μM spin-labeled FKBP. The SLAPSTIC method therefore serves either for some 50-fold reduction in protein requirement or for largely increased clarity in the interpretation of NMR screening data, while still permitting a 3−5-fold reduction in protein concentration.

Assuming fast ligand dissociation rates, the transverse R2 (R1ρ) relaxation rates of ligand protons can be approximated by

$$R2 = (1 - p_b)\cdot R2_{free} + p_b\cdot R2_{bound} + p_b\cdot R2_{para} + R2_{ex} \quad (1)$$

where $R2_{free}$ describes transverse relaxation of the unbound ligand, $R2_{bound}$ describes transverse relaxation due to increased correlation time when bound to the (nonmodified) target protein, and $R2_{para}$ describes additional transverse relaxation due to paramagnetic relaxation enhancement when bound to the spin-labeled target protein. p_b is the percentage of bound ligand. $R2_{ex}$ accounts for exchange broadening in the intermediate exchange regime, which can be neglected for weak (high micromolar) binding affinities. For the most upfield aromatic proton of **1**, the individual rates were determined as $R2_{free} = 0.6\ s^{-1}$, $p_b\cdot R2_{bound} = 0.75\ s^{-1}$, and $p_b\cdot R2_{para} = 12\ s^{-1}$ for 50 μM *p*-hydroxybenzanilid **1** in the absence of protein, in the presence of 60 μM FKBP, and in the presence of 20 μM spin-labeled FKBP, respectively.[11] This corroborates the efficiency of paramagnetic relaxation caused by a spin-labeled protein. The effects may even be more dramatic for other proteins in case the spin label is closer to the ligand binding site, since paramagnetic relaxation effects decay with the sixth power of distance.[4] This is particularly important for small proteins with relatively small $R2_{bound}$.

The desired K_d sensitivity of the SLAPSTIC method can be tuned for each individual case by adjusting the concentration of protein target. Since the observed attenuation of ligand signals depends on the percentage of bound ligand, p_b, ligands have to bind with higher affinity to be detected with lower protein concentration. In this respect, it is noteworthy that for weak binding affinities, the percentage of bound ligand, p_b, for a given affinity depends highly on protein concentration but only slightly on ligand concentration, as long as the ligand concentration is not more that 100 times higher than the protein concentration.[12]

The SLAPSTIC method greatly alleviates the requirement of NMR screening for large amounts of soluble protein. It also relieves the necessity for high ligand concentrations, which had severely limited the nature of compounds to be investigated for protein binding, and had led to automation problems, when compounds precipitated in the course of sample preparation or sample transfer. With the SLAPSTIC method, compound concentrations of 50 μM are sufficient to detect binding within experimental times of 2 min per compound mixture. Since the spin labels also quench protein signals, there is almost no protein background, which can hamper analysis of T1ρ relaxation experiments at short spin lock periods (Figure 2, middle). The SLAPSTIC method is extremely easy to set up and analyze, and is highly amenable to automation.

A drawback of the SLAPSTIC method, as with all methods that detect binding by transfer to the ligand resonances,[2b−g] is its inability to detect strongly binding ligands with slow dissociation rates, since the ligand is in high excess. Therefore, tightly binding ligands ($K_d < 10^{-6}$ M) will produce false negatives. Detailed information on the binding site is not available either, although competition experiments with known ligands can give valuable information. The SLAPSTIC method should therefore be regarded as a primary screening method, and promising hits should be further investigated with HSQC- and NOE-based methods.[2a] They can then be spin labeled themselves to discover second-site ligands.[3] Ideally, both ligands are then linked to form a high-affinity ligand.

In conclusion, the SLAPSTIC method belongs to the most sensitive techniques of NMR screening. It offers a reduction in protein demands by 1 or 2 orders of magnitude compared to T1ρ relaxation experiments using nonmodified protein targets,[2d] and by 2 to 3 orders of magnitude compared to ^{15}N,^{1}H-HSQC-based methods.[2a] The requirement for only 50 μM compound concentrations significantly broadens the nature of compounds that can be screened by NMR. This leads to a higher diversity of ligands to be discovered, and to a broader applicability of NMR screening.

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(11) These numbers are derived from the measured T1ρ relaxation times of 1.7, 0.75, and 0.075 s in the absence of protein, in the presence of 60 μM FKBP, and in the presence of 20 μM spin-labeled FKBP, respectively. With K_d = 1.1 mM, p_b = 0.049 and 0.017, respectively. It follows that $R2_{bound}$ = 15 s^{-1} and $R2_{para}$ = 700 s^{-1}.

(12) Simple calculations, assuming a one-step binding model with a single binding site and a K_d of 1 mM, show that for 1 μM protein concentration, 0.1% ligand is bound at 1 μM ligand concentration, and 0.09% ligand is bound at 100 μM ligand concentration. For 10-fold higher protein concentration, these values increase approximately 10-fold.