Spin Labels as a Tool to Identify and Characterize Protein – Ligand Interactions by NMR Spectroscopy

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NMR spectroscopy based discovery and optimization of lead compounds for a given molecular target requires the development of methods with maximum sensitivity and robustness. It is shown here that organic nitroxide radicals ("spin labels") can be used to boost the sensitivity of NMR spectroscopic screening in drug discovery research. The concept of utilizing spin labels in NMR spectroscopy is summarized, examples for successful first-site and second-site NMR spectroscopic screens are given, and guidelines for linker design are presented.

KEYWORDS:

drug research · NMR spectroscopy · protein modifications · screening · spin labels

1. Introduction

Protein NMR spectroscopy has been successfully applied in the field of structure determination and dynamic characterization of proteins, but has only recently been rediscovered as a more general biophysical technique with a wider variety of applications. In particular, its ability to detect and investigate molecular interactions and to characterize them with atomic resolution has received considerable attention, especially in pharmaceutical research, where potent ligands for drug targets are sought to control or treat a particular disease by influencing the underlying molecular mechanism.

How, then, are compounds with such biological activity discovered? The host of available techniques, such as natural compound screening, high-throughput screening, combinatorial chemistry, or structure-based drug design, has recently been complemented by a method termed "SAR-by-NMR".[1] This method originally aims to build up potent (high-affinity) ligands in a modular way, by linking two low-affinity ligands in such a way that their binding affinities multiply. Since the two individual components bind to the protein target only weakly, they are difficult to identify with conventional assays. Therefore, NMR spectroscopy is employed as an assay to detect ligand binding, and NMR spectroscopic screening has become a reliable and robust method for primary or secondary screening or hit validation in drug discovery research.[2] A variety of such screening methods has been developed in recent years.[2–4]

The advantages of NMR spectroscopic screening ("NMR screening") are its high sensitivity for even weak binding interactions, the straightforward process of setting up the assay, its robustness for not producing false positives, the potential to obtain structural information of the binding interaction, and the ability to identify and structurally characterize the binding of two or more ligands at the same time. The most important drawback of NMR screening is the need for large amounts of protein. Depending on the detection method used, protein amounts ranging from 5 mg of unlabeled protein to 200 mg of ^{15}N-labeled protein are needed for the screening of 1000 test compounds toward a 20 kDa protein target. This makes NMR spectroscopic screening only feasible for well-expressing and soluble protein targets. A key activity in several laboratories is therefore the development of more sensitive and robust methods for the detection of protein – ligand interactions by NMR spectroscopy.

Recently, we introduced organic nitroxide radicals such as TEMPO (2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl; see Figure 1C), so-called "spin labels", to the field of NMR screening, and showed that these paramagnetic substances can significantly help to overcome some of the hurdles of this technique.[5, 6] This article illustrates the concept of using spin labels as a tool to identify and characterize protein – ligand interactions.

2. Basic Theory of Spin Labels

Spin labels have a long history in magnetic resonance spectroscopy.[7, 8] Discovered and synthesized in the early 1960s, they were first applied to biological systems in the late 1960s and 1970s. Most of the applications involved electron spin resonance (ESR), which can give information on dynamic processes in the millisecond to nanosecond range.[9] Systems under investigation were often enzymes, but nucleic acids were also studied. The application of spin labels to NMR spectroscopy was based on the

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increase of relaxation rates of neighboring protons caused by the paramagnetic center, the so-called paramagnetic relaxation enhancement (PRE). This can be used to measure distances up to 20 Å, which are too long to be measured by NOE experiments, or to study the dynamics of peptides or proteins by sampling all conformations that lead to short proton – radical distances.

The magnitude of the paramagnetic relaxation enhancement (PRE) caused by dipolar interactions depends on the square of the gyromagnetic ratios of both involved spins, the inverse sixth power of the interspin distance, and the correlation time. The transverse relaxation rate enhancement, $R2_{para}$, is described by Equation (1), where S is the electron spin, γ_I the proton gyromagnetic ratio, g the electronic g factor, β the Bohr magneton, r the distance between the electron spin and the nuclear spin, ω_I the resonance frequency of protons, and τ_c the correlation time of the vector connecting the electron and nuclear spins.[7, 10]

$$R2_{para} = \frac{1}{15} S(S+1) \frac{\gamma_I^2 g^2 \beta^2}{r^6} \left(4\tau_c + \frac{3\tau_c}{1 + \omega_I^2 \tau_c^2} \right) \quad (1)$$

A similar equation holds for the longitudinal relaxation rate enhancement, $R1_{para}$. Since the electron gyromagnetic ratio is 658 times that of the proton, proton relaxation rates in the vicinity of a paramagnetic center are drastically larger than the corresponding diamagnetic (proton – proton) relaxation rates. It is essentially this factor of 658^2 that we aim to exploit to enhance the detection of ligand binding.

The correlation time of the vector connecting the electron and nuclear spins, τ_c, depends on the rotational correlation time of the protein – ligand complex, τ_r, on the electronic relaxation time, τ_s, and on the lifetime of the complex, τ_m, according to Equation (2).[7, 10]

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_m} \quad (2)$$

The electronic relaxation time, τ_s, plays a key role in paramagnetic systems. For organic nitroxide radicals, τ_s is typically in the order of 100 ns, and therefore much longer than the rotational correlation time, τ_r, which is typically in the order of a few nanoseconds. Since τ_m is usually even longer than τ_s, τ_c is dominated by τ_r, the same correlation time that governs relaxation in diamagnetic systems. Given the high gyromagnetic ratio and the long effective correlation time, organic nitroxide radicals drastically increase relaxation rates of neighboring protons, while having little effect on chemical shifts. Organic nitroxide radicals or spin labels are therefore potent relaxation reagents.

Some paramagnetic transition metals, for example Co^{II}, Ni^{II}, low-spin Fe^{III}, or most lanthanides (with the exception of Gd^{III}), on the other hand, have very short electronic relaxation times in the order of $10^{-13} - 10^{-12}$ s. The electronic relaxation times therefore dominate the effective correlation time of the electron – proton vector, and relaxation rates are only slightly affected by such paramagnetic transition metals. Since the magnetic field produced by the paramagnetic transition metals is anisotropic, it does not average out and leads to significant chemical shift changes of neighboring protons. These paramagnetic transition metals are therefore shift reagents. The effect on chemical shifts depends on the third power of the distance between electron and proton, rather than on the sixth power as for the relaxation reagents.[11]

An additional consequence of paramagnetism is partial alignment of the paramagnetic molecule in the magnetic field, due to its anisotropic magnetic susceptibility.[12] As a consequence, dipolar couplings do not average out completely, and the residual dipolar couplings can be measured by using appropriate NMR experiments. These dipolar couplings have become powerful long-range constraints for structure determination by NMR spectroscopy.[13–15]

In principle, paramagnetic relaxation rates can be as much as 658^2, that is, 430 000, times larger than diamagnetic relaxation rates, and this is the theoretical factor of possible reduction in protein concentration that still allows the detection of protein – ligand interactions. In practise, however, the distance between spin label and proton is considerably larger than between two protons. Since paramagnetic relaxation enhancement effects decay with the inverse sixth power of the distance, a large part of this theoretical enhancement factor vanishes. In the example given below, the detection of ligand binding to FKBP, the closest spin label is at a distance of approximately 12 Å to the ligand, about 4.5 times the distance to neighboring protons. Considering the inverse sixth-power distance dependence and the square dependence on gyromagnetic ratios, the effective relaxation enhancement is then $658^2/4.5^6$, about 50. This value corresponds in fact qualitatively to the experimentally observed relaxation enhancement and is equivalent to the possible reduction in protein concentration.

Clearly, the potential to use spin labels as a means to reduce protein concentration for detection of protein – ligand interactions, given by the factor of 658^2, is tremendous. The sixth-power dependence on electron – proton distance underlines the need to carefully design the residue type which is to be spin labeled. Residues that can be spin labeled include lysine, tyrosine, cysteine, histidine, and methionine.[8, 16] At least one residue of this type should be as close as possible to the binding site, but must not interfere with ligand binding. Availability of the three-dimensional structure or a reliable homology model is obviously highly advantageous for the design of a spin-labeling experiment. In our experience, it is almost always possible to identify residues within 10 – 12 Å from the ligand binding site that can be spin labeled and are not expected to interfere with ligand binding.

3. Applications of Spin Labels in NMR Spectroscopic Screening

3.1. Primary NMR spectroscopic screening by using spin labels: SLAPSTIC

As discussed in several reviews, two distinct strategies can be pursued to detect protein – ligand interactions by NMR spectroscopy: observation of the protein resonances or observation of the ligand resonances.[2–4] Observation of the protein resonances is usually done by means of $^{15}N,^{1}H$ or $^{13}C,^{1}H$ HSQC-type

experiments, while observation of the ligand resonances can, for example, be achieved by T1ρ experiments, which measure the transverse relaxation rates of the ligand in the presence and absence of protein (Figure 1). An unbound compound is a small molecule and therefore relaxes slowly. If it binds to the target protein, however, it behaves like a large molecule and relaxes fast. In an exchanging system, one can observe the resonances of the free ligand, but with a relaxation rate that is a weighted average of the relaxation rates in the free and bound states (see Equation (3)).

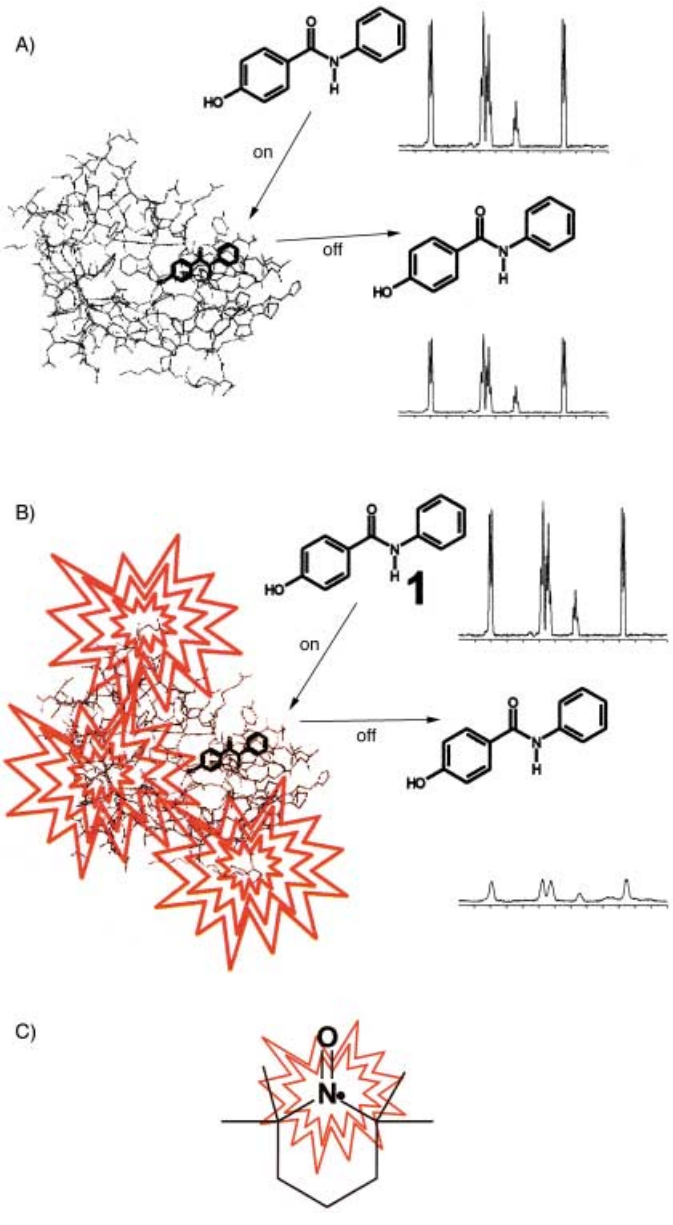

Figure 1. *Principle of the T1ρ experiment (A) and of the SLAPSTIC experiment (B). A) The T1ρ experiment makes use of the increased transverse relaxation rate of the ligand in the bound state, which leads to slightly reduced signal intensity. B) Signal intensity is drastically reduced or completely quenched by paramagnetic relaxation enhancement from the spin-labeled protein in the case of the SLAPSTIC technique. C) A common spin label is TEMPO (2,2,6,6-tetramethyl-1-piperidine-N-oxyl).*

In primary NMR spectroscopic screening, the following reasons often make observation of the ligand resonances preferable:

1. Protein demands are significantly lower, and proteins do not need isotope labeling.
2. The molecular size of the protein is not an obstacle. In fact, most detection methods observing ligand resonances work better for larger proteins.
3. The method is less prone to artefacts caused by slight changes in pH values.[17]
4. If compound mixtures are screened, mixtures containing a hit need not be deconvoluted, but the hit can be identified directly.
5. Poor compound solubility is not an obstacle, as long as it is not below 20 μM (see below).

The drawback of ligand-observing detection methods is that ligand exchange between the bound state and free state must be rapid on the chemical-shift time scale. If the dissociation constant is so low (and binding concomitantly strong) that the ligand does not significantly dissociate during the experiment, the properties of the free ligand are not averaged with the properties of the bound ligand, and it appears as if the ligand does not bind the target protein. The bound resonances of the ligand cannot usually be observed since the protein concentration is typically much lower than the ligand concentration.

In order to test for strong ligand binding by using ligand-detected methods, one can add to the compound under investigation a known weak ligand and observe the relaxation properties of this known weak ligand. If the compound under investigation indeed binds strongly to the protein, it will block all protein binding sites, so that the known weak ligand cannot bind any more and relaxes like a nonbinding compound. This is an indirect proof for strong binding of the compound under investigation.

In Equation (1), it was shown that relaxation of a proton by a paramagnetic moiety is orders of magnitude stronger than relaxation by another proton. In T1ρ experiments, this can be constructively used to further enhance the relaxation rate of the ligand in the bound state. In an exchanging system, the observed transverse relaxation rate, $R2_{obs}$, with a paramagnetic protein target is defined as in Equation (3), where p_b is the fraction of bound protein, $R2_{free}$ is the (small) transverse relaxation rate in the unbound state, $R2_{bound}$ is the (larger) transverse relaxation rate in the bound state, due to the increase in correlation time and spin density, $R2_{para}$ is the (much larger) paramagnetic relaxation enhancement (see Equation (1)), and $R2_{ex}$ accounts for exchange broadening in the intermediate exchange regime, which can be neglected for weak (high micromolar) binding affinities and a large excess of ligand.

$$R2_{obs} = (1 - p_b)R2_{free} + p_b R2_{bound} + p_b R2_{para} + R2_{ex} \quad (3)$$

The potential of the SLAPSTIC method (spin labels attached to protein side chains as a tool to identify interacting compounds) is demonstrated with the FK506 binding protein, FKBP, as a model system. A variety of FKBP ligands are known in the

literature, among them *p*-hydroxybenzanilide (**1**; Figure 1), which binds to FKBP with a dissociation constant, K_d, of 1.1 mM.[18] The structure of FKBP is well-known, and visual inspection showed that several lysine residues are situated within 12 – 15 Å of the active site, without being involved in ligand binding. We therefore chose to spin label lysine residues; this was readily achieved with published protocols.[6]

The benefit of using spin labels for primary NMR spectroscopic screening stems from the occurrence of $p_b R2_{para}$ in the equation for the observed relaxation rate. Figure 2 illustrates the effect of the spin label on the relaxation rates of **1**. This figure shows T1ρ experiments of the mixture of FKBP with ligand **1** and four other aromatic compounds. The upper row shows one-dimensional proton spectra after a short (10 ms) spin-lock period, and the lower row shows one-dimensional proton spectra after a long (200 ms) spin-lock period.[19] In the absence of protein (left column), the signal decay is very small since the transverse relaxation rate of unbound **1** is small. In the presence of 60 μM FKBP (middle column), signals of the interacting compound **1** are partially attenuated due to the term $p_b R2_{bound}$ in Equation (3). In the presence of 20 μM spin-labeled FKBP (by the SLAPSTIC technique), however, paramagnetic effects described by $p_b R2_{para}$ in Equation (3) are so large that the resonances of ligand **1** are completely quenched, and even the resonances of another compound, methyl-4-methoxythiophene-3-carboxylate, which was not known to bind to FKBP and subsequently shown to bind with $K_d = (9 \pm 2)$ mM, are partially attenuated (right column). This demonstrates the high sensitivity of the SLAPSTIC technique.

Knowing the exact concentrations of protein and ligand, as well as the dissociation constant of the complex, the transverse relaxtion rates can be calculated as $R2_{free} = 0.6$, $R2_{bound} = 15$, and $R2_{para} = 700\ s^{-1}$. Paramagnetic relaxation enhancement therefore magnifies the transverse relaxation in the bound state by a factor of about 50, which is in line with theoretical estimates (see above). It should be emphasized that the potential of the SLAPSTIC method is even higher than that: Due to the inverse sixth-power distance dependence of paramagnetic relaxation enhancement [Eq. (1)], paramagnetic effects will be another order of magnitude larger if the average distance of the closest spin label to the binding site is 30% reduced with comparison to FKBP.

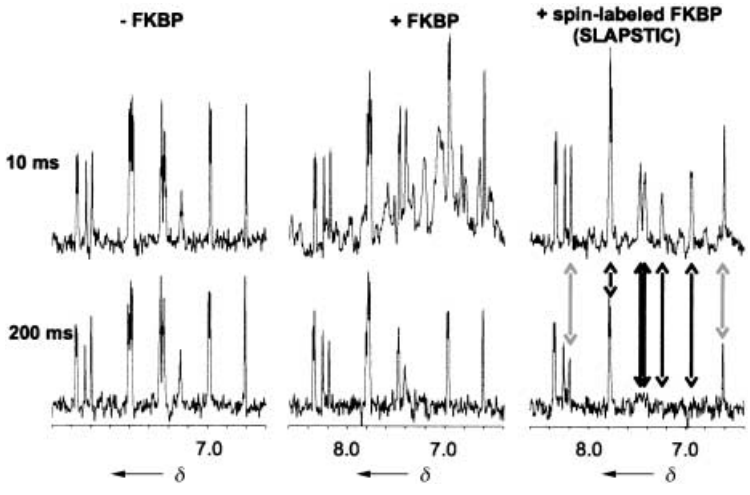

Figure 2. *T1ρ experiments of a mixture of p-hydroxybenzanilide (**1**) and four other aromatic compounds, in the absence of FKBP (left), in the presence of 60 μM FKBP (middle), and in the presence of 20 μM spin-labeled FKBP (SLAPSTIC, right). Spectra in the upper and lower rows correspond to spin-lock periods of 10 ms and 200 ms, respectively. Higher attenuation at 200 ms, as visible in the SLAPSTIC spectra (right), means faster relaxation in the bound state and easier detection of binding. Resonances of **1** are marked with black arrows and resonances of methyl-4-methoxythiophene-3-carboxylate (see text) with gray arrows. The remaining signal at δ = 7.8 comes from another compound.*

3.2. Protein amounts needed for SLAPSTIC screening

The drastic paramagnetic effect on the transverse relaxation rate can be used to make the distinction between binding and nonbinding compounds so clear that analysis of SLAPSTIC experiments can most easily be automated. Figure 2 shows an additional advantage of SLAPSTIC spectra compared to conventional T1ρ experiments: Since the protein resonances themselves are also quenched by the spin label, there is virtually no background signal from the protein, even with short spin-lock periods. Protein background signals are often strongly disturbing when accurately measuring T1ρ relaxation rates (Figure 2, upper middle). The absence of protein background signals and the relatively high sensitivity of T1ρ experiments permit the analysis of even poorly water soluble compounds.

Alternatively, the drastic paramagnetic effect on the transverse relaxation rate can be used to decrease the protein concentration, while maintaining clear discrimination in the transverse relaxation rate. How low can protein concentrations become, while still observing significant paramagnetic relaxation enhancement? The crucial parameter in Equation (3) is p_b, the fraction of bound ligand. If p_b is too low, the observed transverse relaxtion rate, $R2_{obs}$, is dominated by $R2_{free}$, and no sufficient distinction is possible between binding and nonbinding compounds. However, the larger $R2_{para}$, the smaller p_b can get while the distinction is still possible.

Figure 3 shows calculated values for the fraction of bound ligand as a function of ligand concentration, for three different dissociation constants and two different protein concentrations. It is important to realize that the fraction of bound ligand depends almost linearly on protein concentration, and also strongly on binding affinity. The dependence on total ligand concentration is relatively small for weak affinities, and larger for strong affinities. The contribution of paramagnetic relaxation enhancement to the observed transverse relaxation rate is $p_b R2_{para}$. If $R2_{para}$ is tenfold higher due to a well-placed spin label, p_b can be ten times lower with equal observed transverse relaxation rate, $R2_{obs}$. Due to the almost linear dependence of p_b with protein concentration, this allows a tenfold reduction in protein concentration while maintainig the observed transverse relaxation rate.

Alternatively, the protein concentration, and thereby p_b, can be adjusted so that $p_b R2_{para}$ is just large enough for a threshold binding affinity to be detected with SLAPSTIC. Thus, the protein concentration can be tuned for the desired K_d sensitivity of the experiment, so that

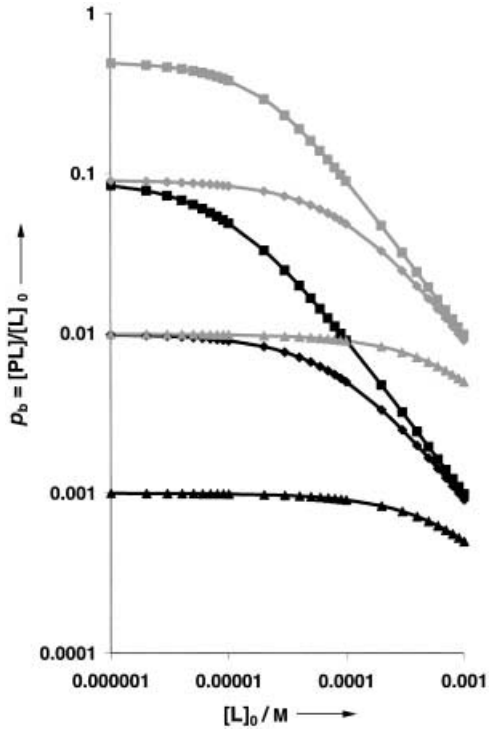

Figure 3. *Calculated ratio of bound ligand ($p_b = [PL]/[L]_0$, where [PL] is the concentration of the protein – ligand complex, and $[L]_0$ is the total ligand concentration) as a function of total ligand concentration. The graph is in double logarithmic scale. The dissociation constants, K_d, are 10 μM (squares), 100 μM (diamonds), and 1 mM (triangles). Protein concentrations are 1 μM (black curves) and 10 μM (gray curves).*

only moderately strong ligands, but not very weak ligands, are detected in the experiment.

3.3. Second-site NMR spectroscopic screening with spin labels

After validation of the primary ligand by other NMR spectroscopic methods such as HSQC or NOESY experiments, the in these cases identification of a second-site ligand, which binds simultaneously with the first ligand at a second, neighboring binding site, is often desired. If both ligands are then linked, the affinity of the linked compound can be much higher than the affinities of the two individual fragments. A nanomolar ligand can then result based on millimolar or micromolar fragments.

Identification of a second-site ligand requires saturation of the first binding site by the first ligand. Unfortunately, this is often not possible due to the weak affinity and poor water solubility of the first ligand. For example, if K_d for the first ligand is 200 μM, and its aqueous solubility is 100 μM, only about 33% of all first binding sites will be saturated. Any test compound in a second-site screen can then bind to the first binding site instead of the desired second binding site and thus produce a false positive response. These false positive hits are extremely difficult and time-consuming to identify, but can be completely removed by employing spin labels, as described in this section.[5]

A true and desired second-site ligand binds to the target at the same time (and therefore at nonoverlapping binding sites) and in the vicinity to the first-site ligand. If the first ligand is spin labeled, a quenching effect of the spin label on other compounds is observed if, and only if, both compounds bind at the same time and in the vicinity (Figure 4). If the putative second ligand actually binds to the first binding site, it will never bind at the same time as the spin-labeled first ligand, and will

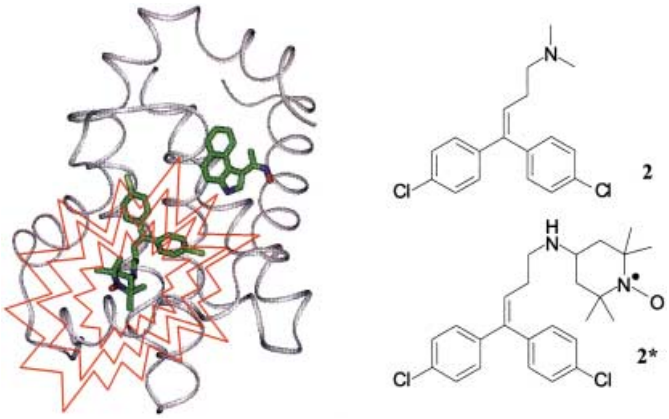

Figure 4. *Principle of second-site screening by using a spin-labeled first ligand. First-site ligand **2** was spin-labeled to yield **2***. The quenching effects of **2*** on the resonances of any second-site ligand are observed.*

therefore not produce a false positive result. If a compound does not bind the target at all, it will accordingly never be in close vicinity to the spin-labeled first ligand. The average distance between a test compound and the spin-labeled first ligand in the absence of target protein is too large for any quenching effects to be observed on the test compound. Only simultaneous binding to the target protein brings the spin-labeled first ligand and the second-site ligand close enough together to observe paramagnetic relaxation enhancement on the second-site ligand caused by the spin-labeled first ligand.

As an example, the identification of a second-site ligand to the anti-apoptotic protein Bcl-xL is shown in Figure 4. Compound **2**, a weak ligand for Bcl-xL ($K_d = 140$ μM), was identified by an enzyme-linked immunosorbent assay (ELISA) based high-throughput screening assay, and second-site ligands were sought by NMR spectroscopic screening to improve its potency after linking. The observation of small, specific chemical shift differences of **2** in the presence and absence of Bcl-xL, knowledge about its structure – activity relationship, and modeling studies, suggested that the aromatic groups are primarily interacting with Bcl-xL, while the substituents of the tertiary amine are noncritical for binding. Therefore, **2** was derivatized with TEMPO at the tertiary amine to yield spin-labeled **2***. The ELISA-based assay confirmed that the binding affinities of **2*** and **2** are not significantly different.

For second-site NMR spectroscopic screening, Bcl-xL and spin-labeled ligand **2*** were incubated with a mixture of eight aromatic compounds, and paramagnetic relaxation enhancement effects from **2*** to any of the compounds were investigated by T1ρ relaxation experiments.[5] Figure 5 shows the corresponding T1ρ spectra, with short and long mixing times in the upper

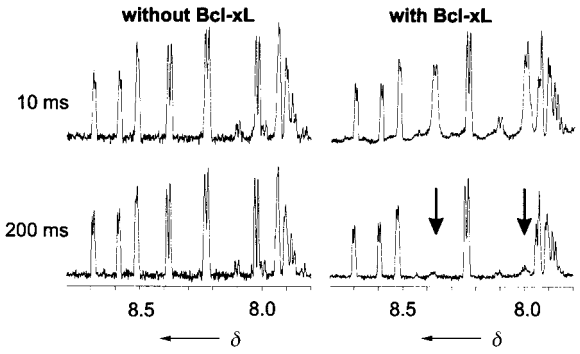

Figure 5. *Identification of a second-site ligand for Bcl-xL by using spin-labeled first ligand **2***. The figure shows T1ρ spectra of a mixture of eight aromatic compounds with spin-labeled compound **2***, in the absence (left) and presence (right) of Bcl-xL.*

and lower row, respectively. The left column was recorded in the absence of Bcl-xL. It serves as a control: None of the compounds experiences paramagnetic relaxation enhancement, since the average distance in diluted solution between noninteracting ligands is large. The right column shows the same spectra in the presence of Bcl-xL. While most compounds still do not experience any paramagnetic relaxation enhancement, the resonances of one compound, marked by the arrows, are completely quenched. This compound is therefore unambiguously detected as second-site ligand.

It should be stressed that there are essentially no sources for false positive detection in this spin-labeling experiment. If a compound experiences paramagnetic relaxation enhancement, it can be safely assumed that the compound binds simultaneously and in the vicinity to the spin-labeled first ligand, and that it is therefore a true second-site ligand.

4. Linker Design

Both the first- and second-site ligands are generally low-affinity ligands with dissociation constants in the micromolar range. They need to be chemically linked in order to obtain a high-affinity, nanomolar ligand. Favorable placement of the linker is crucial for the potency of the linked compound. The optimal linker should satisfy three criteria: First, it should exert little strain on the two individual components, and should allow them to occupy exactly the same binding site in the same orientation in the linked compound as in the unlinked fragments. Second, while allowing the flexibility for the two components to adapt optimal binding orientations, the linker should be as rigid as possible in order to preform the bioactive conformation of the compound, and to reduce the entropic cost of binding.[20] Third, the linker must not have unfavorable interactions with the protein; if it has interactions at all, they should be optimized to affect binding positively.

In the ideal case, the structure of the ternary complex would already have been determined by X-ray crystallography or NMR spectroscopy. This would provide great assistance in designing the linker by molecular modeling. Unfortunately, this ideal case is rather rare. Fortunately, there are several other clues to design a successful linkage by NMR spectroscopy. The atoms on both fragments to which the linker should be attached can be identified by a combination of various techniques:

- Chemical-shift changes or selective line-broadening of the ligand resonances upon addition of protein indicate which parts of the ligand are in contact with the protein. Residues that are unaffected are probably not in contact with the protein, and are therefore candidates for linker attachment. Alternatively, the binding epitopes can be mapped by saturation transfer difference (STD) type experiments.[21]
- In the case of second-site screening with a spin-labeled first ligand, the dependence of the paramagnetic relaxation enhancement on the inverse sixth power of the distance [Eq. (1)] leads to differential quenching effects on the second ligand, depending on its proximity to the paramagnetic center. Those resonances of the second ligand that are most strongly affected are located nearest to the first ligand and are therefore primary candidates for linker attachment. The absolute distance between those protons and the first ligand can be estimated by using Equation (1).
- If site-specific spin-labeled protein is available,[22] quantification of quenching effects on both first-site and second-site ligands can additionally aid in constructing a model of the ternary complex. Site-specific spin labeling can also be achieved by selectively labeling the N-terminal NH_2 group, whose pK_a value is about two units lower than the pK_a of lysine side chains,[23] so that the N terminus is more reactive. This method seems more straightforward than the amino terminal $Cu^{II}(Ni^{II})$-binding (ATCUN) motif method,[24] since it does not require recloning or reexpression of protein.
- In any case, a transfer NOE experiment should be recorded on the ternary mixture between protein, first ligand, and second ligand. Since both the first and second ligand bind weakly to the protein, they are likely to exhibit transferred NOEs that reflect their bound conformation. If both ligands bind in close proximity, that is, if there are protons on both ligands that are within 4 Å in the ternary complex, then these protons can show an interligand NOE effect.[25] In that case, the respective atoms for linker attachment are easily identified, and the required length of the linker can be estimated from the strength of the NOE.[26] If interligand NOEs cannot be identified although transferred NOEs are present, the distance between both ligands may be too large, and it may be worthwhile investigating ligand analogues.

Once the atoms to which the linker is attached are defined and the length of the linker has been estimated, the exact nature of the linker will be designed on the basis of structure – activity relationships of the individual ligands or the structure of a homology model of the protein.

5. Conclusions and Outlook

Spin labels can be used to identify and characterize interactions between proteins and ligands. The main advantages are reduced protein consumption and increased robustness of the experiment against detection of false positives. Spin labels can aid in the identification of ligands by NMR screening, to identify both

first-site ligands by the SLAPSTIC method, and second-site ligands by using spin-labeled first ligands. Both methods have been developed in our laboratory and are now extensively and successfully used. After the identification of first- and second-site ligands, suitable linkers can be designed by the protocols outlined above, with information from interligand NOEs, differential line broadening, and quantification of paramagnetic relaxation enhancement. This procedure can result in the NMR based discovery of nanomolar ligands for a protein target of essentially unlimited molecular size.

The concept of using spin labels to enhance the sensitivity of NMR spectroscopy has not only been used in solution-state, but also in solid-state NMR spectroscopy. In a process called dynamic nuclear polarization, the polarization of unpaired electrons (from spin labels dissolved at high mM concentrations) is transferred to protons of the biomolecule of interest. This enhances the sensitivity of the NMR spectroscopy experiment by up to two or three orders of magnitude.[27] Spin labels will continue to play a dominant role in ESR, solid-state NMR, and solution-state NMR spetroscopies, with growing importance in the area of NMR spectroscopic screening.

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