Using *tert*-Butyl Groups in a Ligand To Identify Its Binding Site on a Protein

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ABSTRACT: Few methods allow determining the binding site of tightly binding ligands. We show that ligands containing a *tert*-butyl (e.g., Boc) group produce easily observable nuclear Overhauser effects (NOE) with the target protein even when the *tert*-butyl group is not highly solvent exposed. NOEs with methyl groups of the target protein are readily assigned by selectively isotope labeling, presenting a practical and quick way to pinpoint the location of the ligand without any prior specific nuclear magnetic resonance assignments of the protein. The approach works for nonexchanging ligands as well as for weakly binding ligands.

KEYWORDS: Boc group, nuclear Overhauser effects, ligand binding site

Nuclear magnetic resonance (NMR) spectroscopy is a widely used tool for drug discovery in the pharmaceutical industries.^{1,2} A frequent scenario is that the crystal structure of the target protein is available but not in complex with the ligand of interest. In this situation, further development of any ligand hinges on determining its binding site on the target and establishing its specificity of binding. In principle, NMR spectroscopy is uniquely positioned to yield structural information on the protein–ligand interaction at atomic resolution, but the sensitivity of chemical shifts with regard to changing chemical environments means that NMR resonance assignments of the protein change in response to different ligands, compromising throughput. Here we present a new strategy, which harnesses intermolecular NOEs with *tert*-butyl groups in the ligand molecule to determine its binding site on the protein. It is applicable also to protein–ligand complexes with tightly bound ligands. Our strategy relies on the crystal structure of the free protein but requires no prior resonance assignments of the protein NMR spectrum.

tert-Butyl groups produce intense signals in the ¹H NMR spectrum that can readily be detected against the background from the numerous NMR signals of a protein. The signals are narrow because splittings due to *J*_{HH} couplings remain unresolved, and the effective correlation times are short due to rapid rotation of the methyl groups and of the *tert*-butyl group itself. The signal of a *tert*-butyl group is also strong, as it corresponds to nine equivalent protons, greatly facilitating its detection. We have previously shown that site-specific incorporation of *O*-*tert*-butyl-tyrosine (Tby) into proteins allows the observation of the *tert*-butyl group in molecular systems as large as 320 kDa without dilution of the proton spin density by perdeuteration, provided the *tert*-butyl group is highly solvent exposed to allow rapid reorientation of the C–H bonds of the *tert*-butyl group.³ In the case of Tby, the

reorientation is promoted further by rotation about the C–O bonds linking the *tert*-butyl group to the rest of the amino acid. Broader ¹H NMR signals are observed, however, when the *tert*-butyl group is only partially solvent exposed. For example, the ¹H NMR signal of the *tert*-butyl group of an inhibitor bound to the 27 kDa dengue virus NS2B-NS3 protease were more difficult to identify in 1D ¹H NMR spectra of paramagnetically labeled samples, ultimately requiring NOE cross-peaks with the *tert*-butyl group for their unambiguous identification.⁴ NOEs also proved necessary for spectral identification of highly solvent exposed *tert*-butyl and trimethylsilyl groups in 95 kDa complexes between single-stranded DNA binding protein (SSB) and single-stranded DNA.⁵

The present work demonstrates the feasibility of detecting intermolecular protein–ligand NOEs when the ligand contains *tert*-butyl groups located in binding pockets of the protein. These NOEs allow analysis of the binding site of the ligand without any prior assignments of the NMR resonances of the protein. While there are many NMR methods for studying protein–ligand complexes of weakly binding ligands, it remains difficult to identify binding sites on the target protein, if the bound ligand does not exchange with its free form on the time scale of the experiment.⁶ The present strategy opens a way for binding site determination that is applicable to tightly binding ligands.

The concept is illustrated using the complex between the hepatitis C virus (HCV) inhibitor asunaprevir (ASV; also referred to as BMS-650032) and the full-length NS3 protein (69 kDa) and its protease domain (NS3pro; 23 kDa), each in a

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construct with an 11-residue polypeptide segment from the cofactor NS4A. ASV in combination with daclatasvir has been shown to be effective against HCV genotype 1b infection in phase 3 clinical trials.⁷ It is a tightly binding ligand, inhibiting the proteolytic activity of HCV NS3 with IC₅₀ values in the low-nanomolar range for a wide range of HCV genotypes.⁸ It contains two *tert*-butyl groups, one of them as part of a Boc group (Figure 1A).

Figure 1. ¹H NMR signals of the *tert*-butyl groups of the inhibitor ASV in complex with HCV NS4A-NS3pro. (A) Chemical structure of the inhibitor ASV. The *tert*-butyl groups 1 and 2 of the inhibitor are highlighted in red and blue, respectively. (B) The 800 MHz 1D ¹H NMR spectra of 50 μM solutions of HCV NS4A-NS3pro in NMR buffer (20 mM sodium phosphate, pH 6.5, 150 mM NaCl, 5 mM DTT, 10% D₂O) at 25 °C. The spectra were recorded in the presence of an equimolar amount of inhibitor (top spectrum) or without inhibitor (bottom spectrum). The red and blue arrows identify the chemical shifts of the *tert*-butyl groups 1 and 2 of the inhibitor.

The nonstructural protein NS3 of HCV encodes a helicase preceded by a serine protease, NS3pro, that functions together with the N-terminal part of NS4 (NS4A) as a cofactor.⁹ Crystal structures have been solved for the complex between ASV and NS4A-NS3pro^{10,11} and for NS4A-NS3 in complex with different inhibitors.^{12,13} Structural information for ASV bound to full-length NS3 is still lacking. The crystal structure of NS4A-NS3pro shows that ASV binds in a pocket of the protease facing the helicase domain. The *tert*-butyl group of the Boc group is about 60% solvent exposed, whereas the other *tert*-butyl group is more buried. The ¹H nuclei of the two *tert*-butyl groups of ASV are as close as 5.5 Å to each other and within 5 Å of the methyl groups of the protein. NMR spectroscopy of NS4A-NS3pro and full-length NS4A-NS3 is made difficult by poor stability of the protein samples against precipitation. In the past, stability was enhanced by the addition of 4% glycerol and 0.1–1.5 mM CHAPS.^{14,15} To demonstrate the performance of our method in difficult cases, we measured in aqueous solution without cosolvents.

The free inhibitor is only poorly soluble in water. In DMSO, the chemical shifts of the *tert*-butyl groups were reported to be 1.10 ppm and about 0.96 ppm.¹⁰ In the complex with NS4A-NS3pro, the ¹H NMR signal of the Boc group (*tert*-butyl group 1, at about 1.35 ppm) is readily detected by comparison with

the 1D ¹H NMR spectrum of the free protein (Figure 1). The second *tert*-butyl group (*tert*-butyl group 2, at about 1.1 ppm) is obscured by overlap with similarly intense signals of the protein (Figure 1B; ultimately, the specific assignments were confirmed by NOEs, see below). The chemical shifts of the *tert*-butyl groups became clear in a NOESY spectrum, where the cross-peaks with the *tert*-butyl groups stood out for their narrow line shape, making them taller than most of the intraprotein cross-peaks. A notably intense cross-peak was observed between the resonances of the *tert*-butyl groups (Figure 2A). The NOEs with methyl groups of the protein yielded the specific assignment of the two *tert*-butyl groups and identification of the ligand binding site on the protein.

Figure 2. NOESY spectra of the complex between HCV NS4A-NS3pro (150 μM) and ASV. The spectra were recorded at 25 °C, using a mixing time of 200 ms. The chemical shifts of the *tert*-butyl groups 1 and 2 are identified by dotted red and blue lines, respectively. NOEs with valine and isoleucine residues and an amide proton are identified. Cross-peaks between the *tert*-butyl groups appear at 1.11 and 1.37 ppm. (A) NOESY spectrum of the complex with unlabeled NS4A-NS3pro. (B) NOESY spectrum of a complex where NS4A-NS3pro was prepared with perdeuterated valine and uniformly ¹³C-labeled isoleucine. Comparison with the spectrum in panel (A) confirms that the *tert*-butyl group 1 makes NOEs with a valine residue. (C) Same spectral region as in panel (B), except of a spectrum recorded without ¹³C-decoupling in the δ₁ dimension. Comparison with the spectrum in panel (B) confirms that the *tert*-butyl group 2 makes NOEs with an isoleucine residue.

Initially, no resonance assignments were available for NS4A-NS3pro or ASV in the complex. Clearly, however, some of the NOESY cross-peaks displayed by the *tert*-butyl groups in the methyl region must arise from intermolecular NOEs with the protein, as ASV contains only a single methyl group beyond the *tert*-butyl groups (Figure 1A). NOEs with methyl groups in the spectral range between 0 and 1 ppm are very likely with isoleucine, leucine, or valine. To identify the residue types, we prepared a protein sample selectively labeled with deuterated valine and ¹³C-labeled isoleucine. In the NOESY spectrum recorded for this sample, three cross-peaks disappeared, which were thus assigned to valine (Figure 2B). Furthermore, two cross-peaks became undetectable when ¹³C-decoupling was

omitted, assigning them to isoleucine (Figure 2C). In this way, a single selectively isotope-labeled sample afforded residue-type specific assignments of the methyl resonances of isoleucine, leucine, and valine (any remaining cross-peaks with methyl groups would have been assigned to leucine).

To test the capability to identify plausible binding sites of ASV on the protein without reference to the crystal structure of the complex, we used the programs RaptorX¹⁶ and COACH.¹⁷ Starting from the amino acid sequence of HCV NS4A-NS3pro as the sole input data, the programs make use of available X-ray structures of homologous proteins to suggest binding sites. Both programs predicted ligand binding in proximity to Ile132, Leu135, and Val158 (Table 1). Indeed, based on the crystal

Table 1. Ligand Binding Site Residues of HCV NS4A-NS3pro Suggested by the Programs RaptorX and COACH^a

RaptorX ¹⁶	COACH ¹⁷
Q41, F43	Q41, T42, F43
H57, G58	H57, G58
L82	V78, D81
R123	R123
<u>I132</u> , <u>L135</u> , K136, G137, S139	<u>I132</u> , <u>L135</u> , K136, G137, S138, S139, G140
F154, R155, A156, A157, <u>V158</u> , C159	F154, R155, A156, A157, <u>V158</u> , D168

^aOnly the amino acid sequence was provided to the respective web servers. The residue numbering follows the convention used in the crystal structure. Underlining identifies the isoleucine, leucine, and valine residues suggested by both programs to be involved in ligand binding.

structure of the NS4A-NS3pro complex with ASV (Figure 3), the ¹H–¹H distances between the *tert*-butyl group 1 and the methyl groups of Val158 are as short as 2.6 Å, and the ¹H–¹H distances between the *tert*-butyl group 2 and the methyl groups of Ile132 are as short as 3.3 Å. Notably, there are few sites on NS4A-NS3pro where methyl groups of isoleucine, leucine, or valine (ILV) are solvent exposed, and there are very few sites where the *tert*-butyl groups of ASV could simultaneously contact methyl groups of different ILV residue types (Figure 3A). With the crystal structure of the complex with ASV in hand, the intermolecular NOEs assign the ¹H NMR signal of the *tert*-butyl group 1 to the Boc group of ASV.

While the characteristic chemical shifts of ILV methyl groups provide a straightforward way of NMR resonance assignment by residue type, NOESY cross-peaks in other spectral regions are much more difficult to attribute to specific residue types. For example, the NOE at about 8 ppm in Figure 2 can be assigned to an amide proton by virtue of its absence from a spectrum recorded in D₂O (Figure S2), but it is difficult to attribute to specific protein residues without residue-selective isotope labeling of many different amino acid types. We did not attempt to do so, as the most likely assignment is the carbamate proton of the ligand next to the *tert*-butyl group 2 (Figure 1A).

To probe the broader applicability of the assignment strategy described above, we recorded a NOESY spectrum of ASV in complex with a 69 kDa construct of full-length NS4A-NS3. The spectrum displayed only a single NOE that could be attributed to the *tert*-butyl groups, namely, the NOE between the *tert*-butyl groups 1 and 2 (Figure 4A). The limited sensitivity of the experiment arose from the low concentration of the complex as well as its limited stability as, in our hands, all NMR samples of the protein–ligand complex were unstable at room temper-

Figure 3. ASV binding site and NOEs with HCV NS4A-NS3pro. The figure shows the crystal structure of the complex between ASV and HCV NS4A-NS3pro (PDB ID: 4NWL; Scola2014). (A) Surface representation of the protein with the bonds of ASV shown as sticks. Isoleucine, valine, and leucine residues are highlighted in cyan, magenta, and blue, respectively, and assigned if they line the ligand binding site. (B) Stereoview of ASV bound to the protease. The protein is shown as gray ribbons. The inhibitor and the amino acid residues Ile132 and Val158 are shown in stick representations. Dashed lines highlight short distances between the *tert*-butyl groups and the methyl groups of Ile132 and Val158. Corresponding ¹H–¹H distances are below 5 Å.

Figure 4. NOEs between the *tert*-butyl groups of ASV in the 69 kDa complex with HCV NS4A-NS3. (A) Region of the NOESY spectrum showing the NOEs between the *tert*-butyl groups. The spectrum was recorded for an approximately 150 μM sample with a mixing time of 200 ms. Red and blue dotted lines indicate the chemical shifts of the *tert*-butyl groups in the complex with NS4A-NS3pro (Figure 2). The change in chemical shifts indicates that the *tert*-butyl groups sense the presence of the helicase domain of NS3. (B) Structure and binding site of ASV in the complex with NS4A-NS3pro superimposed onto the crystal structure of NS4A-NS3 (PDB ID: 5FPS; Saalau2012), showing the inhibitor in a stick representation, NS4A in orange, and the protease and helicase domains in gray and gold, respectively. The helicase domain of NS3 and its C-terminus are close to the inhibitor binding site on NS3pro.

ature, preventing any NMR experiments lasting longer than 12 h. Nonetheless, it is interesting to note that the chemical shifts of the *tert*-butyl groups changed compared with the complex with NS4A-NS3pro (Figure 2A). This is in agreement with the

crystal structures of NS4A-NS3, which show that the binding site of ASV faces the helicase domain (Figure 4B).^{12,13} As expected for the increased molecular weight, the cross-peaks between the NMR signals of the *tert*-butyl groups were broader in the complex with full-length NS4A-NS3. Their continued presence suggests that the helicase domain does not significantly affect the binding mode of ASV for the protease domain.

It is remarkable that the NOE between the *tert*-butyl groups 1 and 2 of ASV was readily observable even in the complex with full-length NS4A-NS3, considering that the crystal structure indicates that the shortest ¹H–¹H distance between these two groups is slightly longer than 5 Å (Figure 3B). Possibly, this result reflects the greater mobility of the Boc group (*tert*-butyl group 1), which may transiently shorten the distance to the *tert*-butyl group 2. The NOESY spectrum, however, showed no evidence for a narrower line shape of the *tert*-butyl group 1 than for the *tert*-butyl group 2 (Figure 2), and the latter is unlikely to have any greater degrees of freedom beyond rotations of the *tert*-butyl group and the methyl groups within (Figure 3B). Fundamentally, the shortening of the effective rotational correlation time effected by such rotations reduces the NMR line widths and raises the maxima of NOESY cross-peaks above the level of the much broader intraprotein cross-peaks. The present work shows that this holds true not only for highly solvent-exposed *tert*-butyl groups but also for *tert*-butyl groups embedded in a ligand binding pocket. NOESY cross-peaks between two *tert*-butyl groups can thus be expected to be especially tall.

One of the *tert*-butyl groups of ASV resides in a Boc group. While Boc groups and *tert*-butyl groups in general are compatible with oral drug intake, they are not frequently found in commercial drugs. Boc groups are, however, very common protection groups in organic synthesis. Compared to synthesis of ligand molecules with ¹³C or ¹⁵N labels, which is difficult and costly, synthetic intermediates with a Boc protection group are much more readily available. The present work shows that a Boc group is suitable for the detection of intermolecular NOEs with a target protein. For detection of intermolecular protein–ligand NOEs in high-molecular weight systems, it may be possible to engineer proteins and ligand molecules in a way that the interaction results in intermolecular NOEs between two *tert*-butyl groups.

In summary, we have developed a practical strategy for assigning intermolecular protein–ligand NOEs that do not depend on prior sequence-specific resonance assignments of the protein and can be applied to tightly binding ligands characterized by slow off-rates. Importantly, *tert*-butyl groups provide the signal intensities required for their assignment in the NMR spectrum, if not from the 1D ¹H NMR spectrum (as in Figure 1B), then from a string of intense NOESY cross-peaks (as in Figure 2). Our results show that, in a 27 kDa protein, the requisite cross-peaks can readily be observed even if the *tert*-butyl group is embedded in a binding pocket with only partial exposure to solvent, limiting its degree of freedom for reorientational motions. Attributing the ¹H NMR signals of methyl groups from the protein to different residue types was achieved with a single sample produced with a deuterated and a ¹³C-labeled amino acid. Intermolecular NOEs between *tert*-butyl groups of the ligand and methyl groups of the protein provide a powerful means to pinpoint the location of the ligand on the protein. Finally, the strategy presented here does not depend on perdeuterated protein samples, which is particularly

important for unstable samples such as the HCV NS4A-NS3 protease and eukaryotic proteins, which are not easily amenable to perdeuteration.

■ ASSOCIATED CONTENT

§ Supporting Information

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Experimental details, amino acid sequences used in the present work, assignment of an NOE with the *tert*-butyl group 2 to an exchangeable amide proton, and ¹³C-HSQC spectrum of NS4A-NS3pro selectively labeled with ²H/¹⁵N-valine and ¹³C/¹⁵N-isoleucine in complex with ASV (PDF)

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W.-N.C. performed the experiments. W.-N.C. and G.O. designed the work and prepared the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; Tby, *O*-*tert*-butyl-tyrosine; HCV, hepatitis C virus; ASV, asunaprevir

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