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COMMUNICATIONS

Published on Web 02/15/2003

Use of Selective Trp Side Chain Labeling To Characterize Protein−Protein and Protein−Ligand Interactions by NMR Spectroscopy

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Over the past few years we have witnessed a change in the usage of NMR, from the classic protein structure determination to applications toward the study of molecular recognition phenomena, either protein−protein or protein−ligand interactions. These studies are usually performed by chemical shift mapping in ^{15}N,^{1}H correlation spectra. In drug discovery, this process has proven especially successful as a technique for lead identification and optimization.[1] For large proteins characterized by a high level of overlap, perdeuteration and 3D HNCO-TROSY[2] could be used. Other simpler alternative solutions were proposed including the observation of solvent exposed amide protons[3a] or methyl groups.[3b−d] Recent studies strongly suggest that a reduced number of residues are responsible for most interaction energy in protein−protein and protein−ligand interactions.[4] Above all, Trp, Tyr, and Arg seem to be the most frequent residues in protein's hot spots;[4] thus, techniques for selective isotope labeling for these residues are decidedly appealing.

In this Communication we report a novel, efficient, and cost-effective method to selectively incorporate specific labels into the side chains of Trp residues in recombinant proteins. Our method is based on the tryptophan biosynthesis, which in *Escherichia coli* is executed by tryptophan synthase, a multienzime protein that catalyzes the conversion of indole-3-glycerol phosphate and serine into tryptophan, through indole as an intermediate (Figure 1).[5] Hence, our idea is to make use of the tryptophan biosynthetic pathway to selectively label its side chain in a cost-effective and straightforward manner by using commercially available [2-^{13}C]- or [4-^{13}C]-labeled indole (Cambridge Isotopes) as precursors. Alternatively, one could also supplement the media directly with [2-^{13}C]- and [4-^{13}C]-Trp also commercially available at higher cost (Cambridge Isotopes). Apart from the cost advantage, indole could be more easily chemically synthesized, which would enable the design of different labeling patterns.

As an application, we show the selective ^{13}C-Trp labeling of the third BIR domain of XIAP protein (BIR3). This domain is believed to play an important role in caspase inhibition, end executioners of apoptosis signal pathway.[6] The BIR3 domain contains four Trp residues, two of which are located in the binding site and are directly involved in the interaction with its natural ligands, features that makes this system a good test bench for our selective labeling. Earlier work with ^{15}N-labeled BIR3[6b] revealed that in the apo form only one ^{15}N,^{1}H Trp side chain cross-peak is observed due to slow dynamics of the binding site region. In contrast, the 2D [^{13}C,^{1}H] HMQC spectrum for [4-^{13}C]-Trp labeled BIR3 clearly shows four distinct cross-peaks, one for each Trp side chain residues (Figure 2A,B). In addition, no scrambling of the ^{13}C label was observed throughout the ^{13}C spectral region, therefore indicating the efficiency and selectivity of our labeling scheme. This was also verified by selective incorporation of unlabeled indole into Trp side chains in otherwise ^{15}N labeled BIR3 (Supporting Information). The yields obtained did not differ from those observed

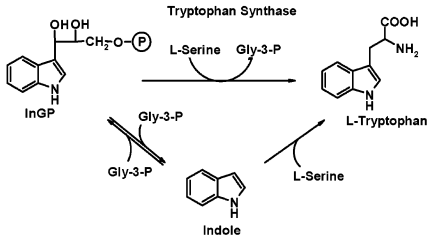

Figure 1. Last two biochemical steps in the Tryptophan biosynthetic pathway.

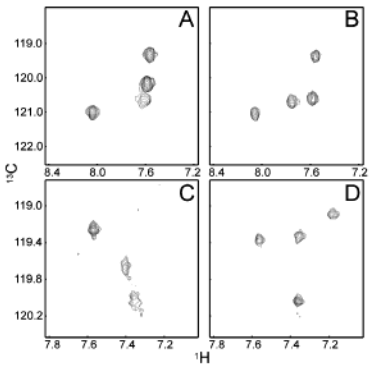

Figure 2. 2D [^{13}C,^{1}H] HMQC (heteronuclear multiple quantum correlation) recorded for [4-^{13}C]- (A and B) and [2-^{13}C]-Trp labeled BIR3 (C and D): (A) Apo [4-^{13}C]-Trp labeled BIR3 and (B) in the presence of 0.4 mM Smac N-terminus 7mer; (C) Apo [2-^{13}C]-Trp labeled BIR3 and (D) in the presence of 0.4 mM Smac N-terminus 7mer. Spectra were recorded with 0.2 mM BIR3 sample in 20 mM phosphate D_2O buffer at pH 7.5 and $T = 30$ °C. The spectra were measured on Varian Unity+ operating at a 500 MHz ^{1}H frequency. 64 × 1024 complex points were acquired with 64 transients per increment leading to a total measurement time of 1 h 30 min per spectrum. Recombinant BIR3 was overexpressed as a His-tag fusion protein using BL21 strain and M9 minimal media. Cells where grown at 37 °C in 2 L shaker flasks until $OD_{600} = 0.8$. Then [2-^{13}C] or [4-^{13}C] labeled indole (Cambridge Isotopes) was added to the media just prior to adding 1mM IPTG for induction (4 h at 37 °C). Then 50 mg of labeled indole was dissolved in 1 mL of DMSO and the solution added to 1 L of media under stirring conditions. Following cell lysis, soluble protein was purified over a His trap chelating column (Amersham, Pharmacia) and thoroughly dialyzed into sample buffer.

by using the same media without indole. Intriguingly, the 2D [^{13}C,^{1}H] spectrum with [2-^{13}C]-Trp BIR3 shows only three signals at 30 °C, one of them considerably broad (Figure 2C), whereas four sharp peaks are visible at 50 °C (Supporting Information). This indicates that slow dynamics, though not as serious as in the case of [^{15}N,^{1}H] HSQC still affects spectra quality for position 2 but not position 4 of Trp side chains. Addition of the reported binder Smac N-terminus 7 residue peptide[6] induces major changes in both spectra. In the case of [2-^{13}C]-Trp labeling, considerable sharpening of signals and appearance of the fourth ^{13}C,^{1}H signal following binding are the most outstanding features, along with remarkable chemical shift changes occurring for another Trp cross-peak. As

10.1021/ja029221q CCC: $25.00

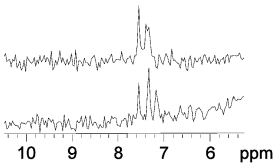

Figure 3. 1D ^{13}C filtered ^{13}C decoupled ^{1}H NMR spectra with [2-^{13}C]-Trp labeled BIR3 in the absence (top trace) and presence (bottom trace) of an inhibitor. The spectra were measured with 200 μM protein concentration with 96 transients on a Varian Unity+500 (total measurement time $\sim$ 3 min).

for [4-^{13}C]-Trp labeled sample, signals are in general better defined and changes are not as dramatic as in the 2-^{13}C case with possibly two Trp cross-peaks being perturbed by the peptide binding. All in all these observations are in agreement with structural studies with BIR3 in complex with the N-terminus Smac peptide.[6]

The examples reported clearly demonstrate that interesting applications for these labeling approaches can be anticipated, ranging from binding studies to side chain dynamics. The first and more obvious application for these labeling schemes would be in the drug discovery field. Given the high occurrence of tryptophan in protein's hot spots, this selective labeling would afford a simple and fast way for compound screening, even with proteins where structural information is not available. In this sense, a small amount of protein and a 1D ^{13}C filtered−^{13}C decoupled ^{1}H NMR experiment measured in the presence and absence of a given ligand (or mixture of ligands) would suffice to detect binding close to a relevant tryptophan. This is illustrated in Figure 3 which shows the effect of the addition of an inhibitor on a 1D ^{13}C filtered−^{13}C decoupled ^{1}H NMR spectrum with [2-^{13}C]-Trp labeled BIR3.

Perdeuteration and aromatic TROSY[7] would further extend the application of these labeling schemes to larger proteins. Hence, the chemical shift perturbation method and/or cross-saturation experiments,[8] detecting saturation transfer to Trp side chains, could be applied to problems such as protein−protein interactions without the complexity that unspecific labeling (and spin diffusion) entails. This is especially true for the 2-^{13}C labeling scheme. In this case perdeuteration would efficiently isolate this spin system from surrounding protons, therefore minimizing relaxation phenomena mainly due to adjoining protons. For these reasons, the [2-^{13}C,^{1}H] moiety in Trp side chains represents also an ideal spin system to detect and measure side chain dynamics of binding site residues. Our studies with [2-^{13}C]-Trp labeled BIR3 revealed that this spin system is very sensitive to solvation and temperature (Supporting Information), probably enhanced by the presence of the neighboring exchangeable proton. This fact enables the measurement of rates of exchange and temperature coefficients, which shed some light on the involvement of Trp side chains in hydrogen bonding, which is often the basis for specific intermolecular interactions.[4]

Finally, tryptophan has also been reported to be important during protein folding, providing key interactions in its early stages.[9] In this respect the use of non-natural fluorinated Trp and ^{19}F 1D NMR to study the contribution of tryptophan side chain in the folding process has been proposed.[10] We believe that the above-described labeling schemes afford a noninvasive approach to deal with these kinds of problems.

In most of the above-mentioned applications, residue-specific Trp resonance assignments would not necessarily be needed, as for example in ligand screening or folding studies. However, the assignments could be obtained by chemical shift mapping with known ligands, detection of ligand-Trp NOEs in ^{13}C-edited [^{1}H,^{1}H] NOESY, or single point mutation followed by selective labeling. In most cases, a single Trp residue is found in the binding site and at most one or two other residues are found elsewhere in the sequence, largely simplifying the assignment problem.

In conclusion, we report selective labeling and NMR observation of Trp side chain nuclei. The scheme is as cost-effective and straightforward as ^{15}N uniform labeling and tries to tackle spectrum complexity and possibly unfavorable dynamic motions while keeping significant information such as ligand and/or protein binding, protein folding, and side chain rates of exchange and dynamics in protein's hot spots.

Acknowledgment. We thank Dr. Martin Renatus and Hwain Shin for the plasmids encoding for BIR3 and Smac and Dr. John Reed for stimulating discussion. We also thank the Spanish Ministry for Education for a fellowship to R.A.R.-M.

Supporting Information Available: Figures S1 and S2 showing deuterium and temperature effects, respectively, on the spectra of [2-^{13}C]-Trp BIR3 (PDF). Figure S3 showing selective Trp side chain ^{14}N labeling in ^{15}N labeled BIR3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA029221Q