Selective Incorporation of ^{19}F-Labeled Trp Side Chains for NMR-Spectroscopy-Based Ligand–Protein Interaction Studies

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In the last few years NMR spectroscopy has increasingly been used in pharmaceutical drug discovery and development as a result of its ability to provide structural and dynamic information at atomic resolution.[1] However, the attainment of structural information on protein–ligand complexes by NMR spectroscopy is limited to small to medium size proteins (MW $<$ 30 kDa) and by the lengthy nature of the protein resonance assignment process.

Nonetheless it is known that protein binding sites often contain only a small subset of residues—mainly tryptophan (Trp), tyrosine (Tyr), and arginine (Arg).[2] This fact has led to a growing interest in the search for new techniques for the selective isotope labeling of these residues. For example, a new labeling method to selectively incorporate ^{13}C at position 2 or 4 of Trp side chains has very recently been reported and has been successfully applied to the study of protein–ligand interactions by use of 2D [^{13}C,^{1}H] correlation spectra.[3] Herein, we report a simple and effective method to selectively label Trp side chains with ^{19}F at position 5 or 6. Until now, ^{19}F-Trp has generally been introduced into proteins by using *Escherichia coli* strains auxotrophic for Trp and by including the amino acid analogue in the growth medium.[4–8] This method requires the time-consuming extra steps of cloning the protein of interest into these strains, which may result in a loss in yield and poor incorporation levels.[9] Instead of employing auxotrophic strains, we find that 3-β-indoleacrilic acid (IAA) (Figure 1 a) can be used as an inhibitor of tryptophan biosynthesis.[10] Addition of IAA (50 mg L^{-1}) to minimal media results in the complete inhibition of *E. coli* cell growth and the inhibition is reversed by L-Trp (50 mg L^{-1}) (Figure 1 b). This observation is in agreement with the accumulation of indole observed in bacterial culture media

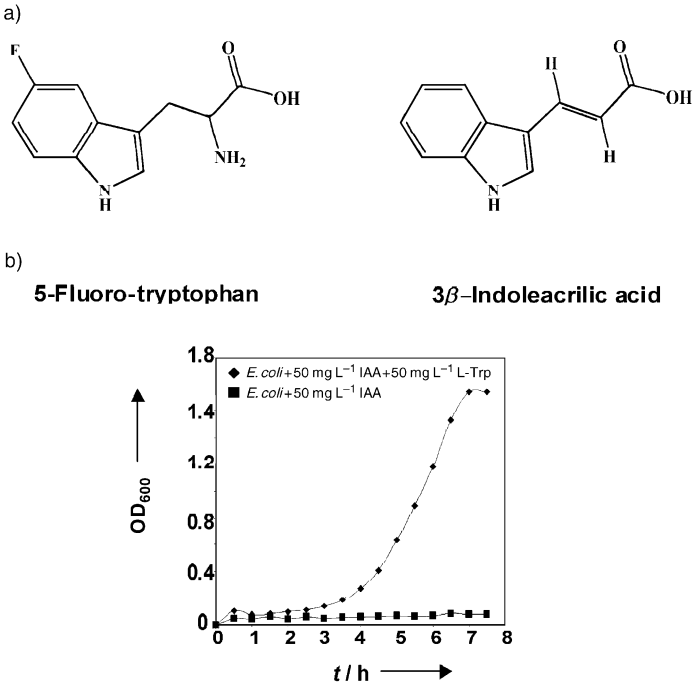

Figure 1. *a) Chemical structures of 5F-Trp and IAA. b) Inhibitory effect of IAA on* E. coli *cell growth.*

when IAA is included as a supplement.[10] Therefore, IAA can be used to block Trp biosynthesis and enhances the incorporation of ^{19}F-Trp, as described below, without the need for auxotrophic strains.

As an application, we describe herein the selective 5F-Trp labeling of the third Baculovirus inhibitor of apoptosis repeat domain (BIR3) of the protein inhibitor of apoptosis XIAP.[11] To incorporate 5F-Trp into the protein, *E. coli* cells were grown in minimal M9 media and then the fluorinated amino acid together with IAA was added just before induction with isopropyl-D-thiogalactopyranoside (IPTG). After protein purification, the extent of the incorporation was tested by means of NMR spectroscopy and MALDI mass spectrometry.

We compared the 1D ^{19}F NMR spectrum of BIR3 with that of the protein complexed with the N terminus of the Smac peptide[12](Figure 2). In agreement with our previous studies with [2-^{13}C]Trp BIR3,[3] three broad signals are present in the ^{19}F NMR spectrum of the apo form, which indicates that slow internal dynamics also affect ^{19}F resonances.[3] When the Smac peptide (2:1 ratio) is added, the spectrum shows significant sharpening of the lines and four separate signals appear, one for each tryptophan residue present in the protein. This change in the spectrum clearly reflects a decrease in the Trp rotational degrees of freedom as a result of peptide binding, similar to the decrease we previously found for [2-^{13}C]Trp BIR3.[3]

The mass spectrum of the obtained 5F-Trp BIR3 shows that its molecular weight is increased by 4×18 amu compared with wild-type protein, which is consistent with the incorporation of four 5F-Trp residues (see the Supporting Information).

The yields for 5F-Trp BIR3 are about half of those obtained for the unlabeled protein expressed under similar conditions.

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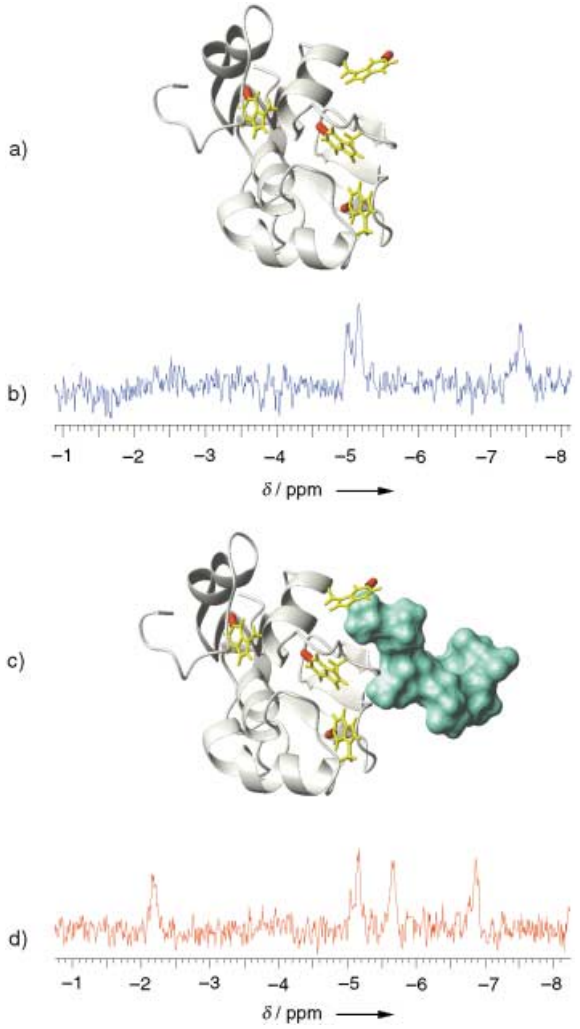

Figure 2. *Ribbon drawings of the apo (a) and complexed (c) forms of BIR3 (Protein Data Bank (PDB) code 1G3F); 5F-Trp side chains are shown. 1D ^{19}F NMR spectrum of BIR3 without (b) and with (d) the Smac peptide.*

In conclusion, we have presented a new method for the simultaneous incorporation of ^{19}F into all Trp side chains of a protein. The introduction of the fluorinated amino acid residue into protein hot spots allows the use of highly sensitive ^{19}F NMR spectroscopy to study, in a very simple and fast way, protein–protein binding events, or to screen large libraries of compounds for applications in drug discovery. Our labeling method is inexpensive and of general applicability and can be employed even for larger proteins and when structural information on the binding site is not accessible. We thus believe that this technique will prove very useful in the drug-discovery field.

Methods

Inhibition of Trp biosynthesis by 3-β-indoleacrilic acid: The inhibitory effect of IAA on cell growth was tested by growing *E. coli* BL21 strain cells in M9 minimal media, in the presence of IAA (SIGMA, 50 mg L^{-1}) and with or without L-Trp (SIGMA, 50 mg L^{-1}). IAA and L-Trp were added to the media dispersed in dimethyl sulfoxide (1 mL), with stirring. The cells were grown at 37 °C in 2-L shaker flasks. To monitor cell growth, the optical density at 600 nm (OD_{600}) was measured every 30 minutes for seven hours. As a further control, we also verified that the growth inhibition by IAA could be reversed by L-Trp.[7] IAA-treated cells (no growth after 7 h) were grown overnight after addition of L-Trp (50 mg L^{-1}) and reached a final OD_{600} of 1.6. The experiments were repeated twice under the same conditions to confirm the reproducibility of our data.

Expression and purification of 5F-Trp BIR3: Recombinant BIR3 was expressed as a His-tag protein in BL21 strain *E. coli* cells and M9 minimal media. Cells were grown at 37 °C in 2-L shaker flasks until $OD_{600} = 1$. At this point, two different expression procedures were carried out. Just before induction, 5F-Trp (SIGMA, 50 mg L^{-1}) and IAA (SIGMA, 10 mg L^{-1}) were added to one aliquot medium (1 L), while only 5F-Trp (SIGMA, 50 mg L^{-1}) was added to another aliquot of medium (1 L). Induction (4 h, 37 °C) was achieved with IPTG (1 mM). Following cell lysis, His-tagged soluble protein was purified over a Hi-trap chelating column (Amersham, Pharmacia) and dialyzed into sample buffer.

^{19}F NMR spectroscopy and mass spectrometry: ^{19}F NMR spectra were acquired on a Bruker 600-MHz spectrometer equipped with a 5-mm ^{19}F/^{1}H probe. Spectra were collected at 25 °C with 0.1-mM samples of ^{19}F-Trp labeled BIR3 in H_2O/D_2O (90:10) phosphate buffer (40 mM, pH 7.5). The spectral width was 11.26 MHz; 16 K data points and 256 scans were collected for a total measurement time of approximately 10 minutes per experiment. For processing, we used the software Mestrec,[13] and a 10-Hz line-broadening weighting function was used. Internal trifluoroacetic acid (0.0 ppm) was used as the reference.

MALDI mass spectra were recorded with a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems).

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[1] M. Pellecchia, D. S. Sem, K. Wüthrich, *Nat. Rev. Drug Discovery* **2002**, *1*, 211–219.
[2] A. A. Bogan, K. S. Thorn, *J. Mol. Biol.* **1998**, *280*, 1–9.
[3] R. A. Rodriguez-Mias, M. Pellecchia, *J. Am. Chem. Soc.* **2003**, *125*, 2892–2893.
[4] E. E. Pratt, C. Ho, *Biochemistry* **1975**, *14*, 3035–3040.
[5] F. Sixl, R. W. King, M. Bracken, J. Feeney, *Biochem J.* **1990**, *266*, 545–552.
[6] J. B. A. Ross, A. G. Szabo, C. W. V. Hogue, *Methods Enzymol.* **1997**, *278*, 151–190.
[7] M. H. J. Seifert, D. Ksiazek, M. K. Azim, P. Smialowski, N. Budisa, T. A. Holak, *J. Am. Chem. Soc.* **2002**, *124*, 7932–7942.
[8] C. Minks, R. Hubert, L. Moroder, N. Budisa, *Biochemistry* **1999**, *38*, 10649–10659.
[9] H. Kim, J. A. Perez, S. J. Ferguson, I. D. Campbell, *FEBS Lett.* **1990**, *272*, 34–36.
[10] P. Fildes, *Br. J. Exp. Pathol.* **1945**, *26*, 416.
[11] Q. Deveraux, J. Reed, *Genes Dev.* **1999**, *13*, 239–252.
[12] Z. Liu, C. Sun, E. T. Olejniczak, R. P. Meadows, S. F. Betz, T. Oost, J. Herrmann, J. C. Wu, S. W. Fesik, *Nature* **2000**, *408*, 1004–1008.
[13] C. Cobas, J. Cruces, F. J. Sardina, MestRe-C 2.3, MestRe-C nD 1.1.1; http://qobrue.usc.es/

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