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APPLICATION NOTE 19

In Vitro Protein Synthesis of Perdeuterated Proteins for NMR Studies

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It is well documented that high levels of deuteration are indispensable for solution NMR studies of polypeptides in structures of sizes above 40 kDa (Fiaux, et al., 2002; LeMaster, 1989; Pachter, et al., 1992). In addition to studies on protein structure and dynamics, obtaining a perdeuterated background is of potential interest for studies of protein functions using residue-selective stable isotope labeling. Unfortunately, the yield of expressed protein *in vivo* in $^{2}H_{2}O$-based media are often significantly lower than those obtained in $H_{2}O$-based growth media, even after lengthy "training" of the cells.

Cell-free protein synthesis can provide for efficient incorporation of selectively labeled amino acids into polypeptide chains in situations where *in vivo* protein expression typically results in isotope scrambling or isotope dilution (Kigawa, et al., 1995; Ozawa, et al., 2004; Waugh, 1996), and its use can extend to cytotoxic proteins, such as proteases or apoptosis-related proteins (Adrain, et al., 2006). So far, cell-free protein synthesis protocols for uniformly deuterated proteins typically yield low non-uniform deuteration levels. This application note summarizes work (Etazady, et al., 2007) using cell-free synthesis methods employing both $H_{2}O$-based and $^{2}H_{2}O$-based *E. coli* cell-extracts for designed, non-uniform incorporation of ^{2}H, ^{15}N-labeled amino acids. NMR results are presented for the 14 kDa FK 506-binding protein (FKBP) and GroEL, an 800 kDa E. coli chaperonine oligomeric protein with 14 identical subunits (Xu, et al., 1997).

Materials and Methods

Cell-free protein synthesis

Analytical-scale cell-free synthesis of FKBP and GroEL in batch mode was used to optimize the reaction conditions of temperature, salts and amino acid concentrations using unlabeled amino acids (Cambridge Isotope Laboratories, Inc., CIL). The optimized conditions were then used for preparative-scale synthesis of U-^{2}H, U-^{15}N-labeled GroEL, using a [U-^{2}H, 98%; U-^{15}N 98%]- amino acid mixture (CIL #DNLM-6818). The large-scale reaction was carried out either in the batch mode or by continuous-exchange cell-free (CECF) protein synthesis. The nutrient compositions used for the batch and CECF modes are given elsewhere with each amino acid at a concentration of 1.5 mM (Etazady, et al., 2007).

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Collection of NMR Data

The 2D [^{1}H,^{1}H]-NOESY experiments with FKBP were recorded at 25°C on a Bruker DRX600 spectrometer equipped with a standard triple-resonance probehead. The protein concentration was adjusted to 0.8 mM. A mixing time of τ_m = 100 ms was used. 128 transients were added with an interscan delay of 1s, resulting in a total measurement time of 20 h. 1024 complex points were recorded with an acquisition time of 128 ms, and prior to Fourier transformation the FID was multiplied with a 75°-shifted sine bell and zero-filled to 2048 complex points. In the ω1(^{1}H)-dimension, 256 complex points were measured, with a maximal evolution time of 32 ms, and the data was multiplied with a cosine function and zero-filled to 512 complex points before Fourier transformation.

For GroEL produced by cell-free expression, a 2D [^{15}N,^{1}H]-CRIPT-TROSY spectrum was recorded at 35°C on a Bruker Avance900 spectrometer equipped with a standard triple-resonance probehead. The protein concentration was 0.7 mM in monomers, and the transfer time was T = 1.4 ms (Fiaux, et al., 2002). 4048 transients were added with an interscan delay of 300 ms, resulting in a total measurement time of four days. In the ω_2 (^{1}H)-dimension, 1024 complex points were recorded with an acquisition time of 81 ms. Prior to Fourier transformation the FID was multiplied with a cosine function and zero-filled to 2048 complex points. In the ω_1 (^{15}N)-dimension, 100 complex points were measured, with a maximal evolution time of 20 ms, and prior to Fourier transformation the data was multiplied with a 20°-shifted sine bell and zero-filled to 256 complex points.

For GroEL prepared *in vivo*, a 2D [^{15}N, ^{1}H]-CRIPT-TROSY experiment was recorded with the same conditions as for the cell-free preparation, except for the following: the experiment was recorded on a DRX750 spectrometer, the protein concentration was 1.9 mM in monomers, 512 transients were added, the acquisition time was 97 ms, in the ω_1 (^{15}N)-dimension the maximal evolution time was 22 ms and the total measurement time was 12 hours.

In all experiments, the baseline was corrected using the IFLAT method (Bartels, et al., 1995) in the direct dimension, and with polynomials of second order in the indirect dimension.

Results and Discussion

Preparation of deuterated S30 extract (D-S30) for cell-free synthesis

The cell extract in ^2H_2O was obtained with reasonable buffer exchange costs using an optimized filtration procedure (Etazady, et al., 2007). For preparative-scale production of D-S30 cell extract, six filtration cycles with a dilution factor of two were used, resulting in a ^2H_2O level above 98%. A higher number of filtration cycles resulted in reduced translation efficiency of the D-S30 extract. In spite of inevitable residual, H_2O content in the other components of the cell-free reaction setup, the final H_2O concentration in the D-S30-based reaction mixture was less than 5%.

The translation efficiency of the D-S30 extract was about 65% of that of the S30 extract in H_2O. The D-S30 extract supplemented with purified ribosomes prior to the final filtration step had a further increased overall efficiency of the synthesis reaction to about 85% of the efficiency of the S30 extract in H_2O without ribosome supplementation.

Cell-free expression of FKBP in H_2O- and D_2O-based growth media

^{2}D[^{1}H, ^{1}H]-NOESY spectra for FKBP produced *in vitro* using D-S30 cell extract and H_2O-based S30-extract are presented in Figures 1 and 2. These and other NMR experiments confirmed the observation of partial back-protonation at the α- and β-positions. Estimates for the degree of back-protonation for amino acids are listed in Table 1.

GroEL synthesis with D-S30 extract

The translation efficiency of the new D-S30 extract for the synthesis of GroEL with unlabeled or with perdeuterated amino acids was similar. The purification elution profiles were similar to those obtained for protonated GroEL (data not shown). An MS analysis of GroEL synthesized from deuterated amino acids in D-S30 extract showed a deuteration level of about 95%.

2D [^{15}N,^{1}H]-CRIPT-TROSY spectra of GroEL synthesized with the D-S30 extract (Figure 3b) and of GroEL expressed *in vivo* (Figure 3a) are similar to one another, indicating that proper assembly of the cell-free synthesized GroEL monomers into the biologically active 14-mer was achieved.

A difference was observed for the peaks contained in the red boxes of Figure 3b, which have higher intensity than the signals at the same chemical shifts in the *in vivo*-produced GroEL (Figure 3a), indicating reduced flexibility of the C-terminal polypeptide segment in GroEL produced *in vivo* (Etazadi, et al., 2007).

Conclusions

A ^2H_2O-based *E. coli* cell extract for use with CIL [U-^{2}H, 98%; U-^{15}N, 98%]- labeled amino acids was developed which enables efficient production of proteins with high deuteration levels (e.g., 95%) for all non-labile hydrogen atom positions. With this approach, *in vitro* synthesis of perdeuterated proteins should be economically viable. In addition to the production of perdeuterated proteins, the protocol may be adaptable for more sophisticated isotope-labeling schemes, which are not readily accessible by other techniques. For example, selective incorporation of individual ^{1}H, ^{13}C , ^{15}N-labeled amino acid types into proteins on a uniform ^{2}H, ^{12}C, ^{14}N background could open new perspectives for active site screening of enzymes and receptor proteins, with practical applications in drug discovery and drug design projects. The approach used here with *E. coli* cell extracts should in principle also be applicable with other cell-free systems, for example, with wheat germ or insect cell extract for the production of eukaryotic proteins.

Amino Acids	Relative degree of α- and β-back protonation[a]
Asp, Asn, Gln, Glu	very high
Ala, Cys, Gly, Phe, Ser, Trp, Tyr	high
Arg, Ile, Leu, Lys, Val	medium
Thr, His, Met	low

Table 1. Amino-acid dependent back-protonation at the α- and β-positions during cell-free protein synthesis using perdeuterated amino acids in H_2O-based culture medium. [a]The classification is based on semi-quantitative analysis of 3D ^{15}N-resolved [^1H, ^1H]-TOCSY and 2D [^1H, ^1H]-NOESY spectra.

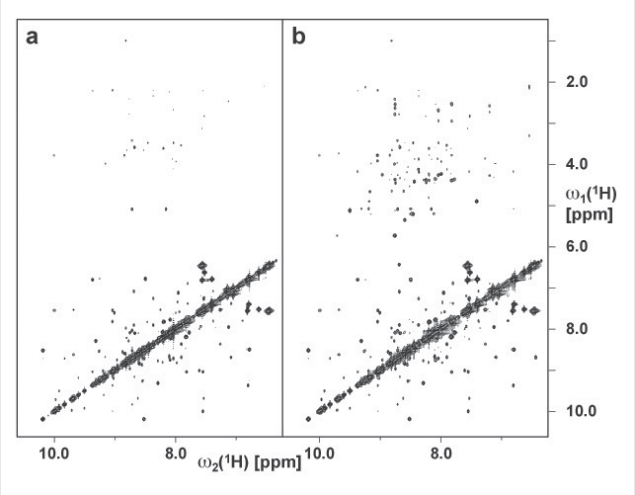

Figure 1. Back-protonation of perdeuterated amino acids during *in vitro* synthesis of FKBP monitored by comparison of the 600 MHz 2D [^1H, ^1H]-NOESY spectra of FKBP produced *in vitro* using either D-S30 cell extract (a) or H_2O-based S30-extract (b). The protein concentration was 800 µM in both samples, the measurements were carried out at 23°C with a mixing time of 100 ms and the two spectra were recorded and processed identically.

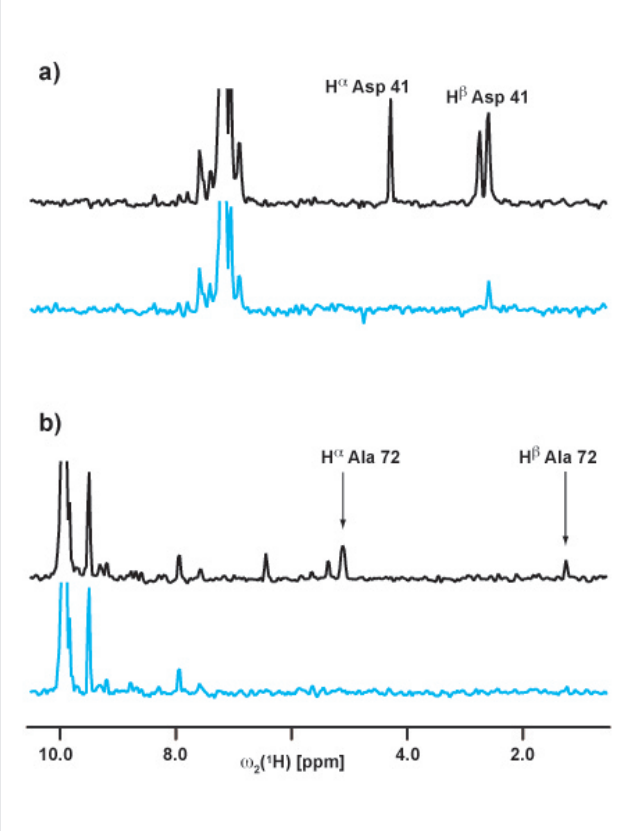

Figure 2. Amino acid-specific back-protonation monitored by comparison of 1D cross-sections taken from the 2D [^1H, ^1H]-NOESY experiments in Figure 1 recorded with FKBP. (a) Asp 41. (b) Ala 72. Color code: black – sample prepared in H_2O-based S30 cell extract; cyan – synthesis in D-S30 cell extract. In both experiments, U-^2H 98%, U-^{15}N 98% amino acids were used in the reaction mixture.

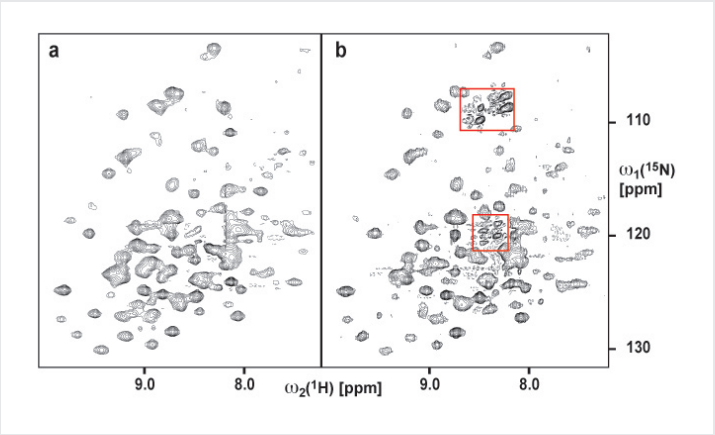

Figure 3. 2D [^{15}N, ^1H]-CRIPT-TROSY NMR spectra (Fiaux, et. al., 2002; Riek, et, al. 2002) of two different samples of uniformly ^2H, ^{15}N-labeled GroEL. (a) Protein expressed *in vivo* in *E. coli* cells. (b) Protein synthesized *in vitro* with D-S30 extract. Two spectral regions highlighted in spectrum (b) by red rectangles identify local differences between the two spectra that are
discussed in the text.

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