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APPLICATION NOTE 36

Cell-Free Protein Synthesis with $^{2}H/^{15}N/^{13}C$-Labeled Amino Acids in H_2O for the Production of Perdeuterated Proteins with ^{1}H in the Exchangeable Positions

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Perdeuteration is essential for NMR studies of big and immobile proteins (> 40 kDa) to slow down the relaxation rates of the remaining ^{1}H NMR signals. Uniformly $^{2}H/^{15}N/^{13}C$-labeled proteins are typically produced by growing *E. coli* in minimal medium with $^{13}C_6,D_7$-glucose as the only carbon source, ^{15}N-ammonium salts as the only nitrogen source, and perdeuteration achieved simply by making up the medium with D_2O instead of water. As the resulting protein product is uniformly labeled with ^{2}H, observation of the backbone amides by ^{1}H NMR requires prior ^{2}H-^{1}H back-exchange of the amide hydrogens. This poses a dilemma for proteins that do not tolerate ^{2}H-^{1}H back-exchange. For example, the hydrogen-exchange of amide deuterons buried in the interior of the protein depends on local (for some amides even global) unfolding of the protein to expose the amides to the solvent. Proteins for which back-exchange is difficult include the large class of proteins that cannot be reversibly denatured (usually leading to precipitation) and proteins of limited stability that don't tolerate long incubation times. Here we illustrate the finding that back-exchange is redundant if the protein of interest is produced by cell-free synthesis from perdeuterated amino acids in H_2O. Importantly, the costs associated with cell-free synthesis compare favorably with those of *in vivo* expression.

A Brief Description of Cell-Free Protein Synthesis

Cell-free protein synthesis uses a cell extract rather than live cells to produce the protein of interest in a coupled transcription-translation reaction. Using cell extracts has numerous advantages: (i) cell extracts are depleted of the DNA from the original organism so the synthesis machinery only produces the target protein encoded by the DNA that is supplied to the reaction mixture; (ii) the process of preparing the cell extract inactivates a number of enzymes that perform chemical transformations between amino acids *in vivo*, therefore metabolic conversions between different amino acids are suppressed; (iii) the chemical environment in which the protein synthesis proceeds can readily be controlled and modified; and (iv) proteins can be produced from linear DNA amplified by PCR making it very easy to introduce mutations by site-directed mutagenesis.[1] Cell-free protein synthesis became an important tool in NMR spectroscopy in 1999 when Kigawa and co-workers established conditions for high-yield protein expression.[2] Cell extracts can readily be prepared from *E. coli* by breaking the cells, centrifugation and collection of the supernatant. In our hands, homemade S30 extracts routinely sustain the production of about 1 mg of protein per mL of reaction mixture. The protocol involves the use of a dialysis system in which the reaction mixture containing the S30 extract is placed in a dialysis bag and immersed in an outer buffer that supplies low-molecular-weight compounds to sustain the protein synthesis reaction, such as amino acids, nucleotides, and ATP. The target DNA is added to the reaction mixture together with any other macromolecules that aid in protein production, such as tRNA and RNA polymerase. The DNA can be the same plasmid DNA that directs the protein expression in conventional *in vivo* systems or it can be PCR-amplified DNA. Detailed protocols for the preparation of S30 extracts and cell-free reactions that we have established in our laboratory have been published.[3]

Cell-Free Synthesis of Perdeuterated Proteins in H_2O

Producing perdeuterated proteins by cell-free synthesis from perdeuterated amino acids in H_2O can be compromised by undesired residual activity of metabolic enzymes in the cell extract. For some amino acid types, these metabolic activities can substitute some of the carbon-bound deuterons by protons. The effect is most

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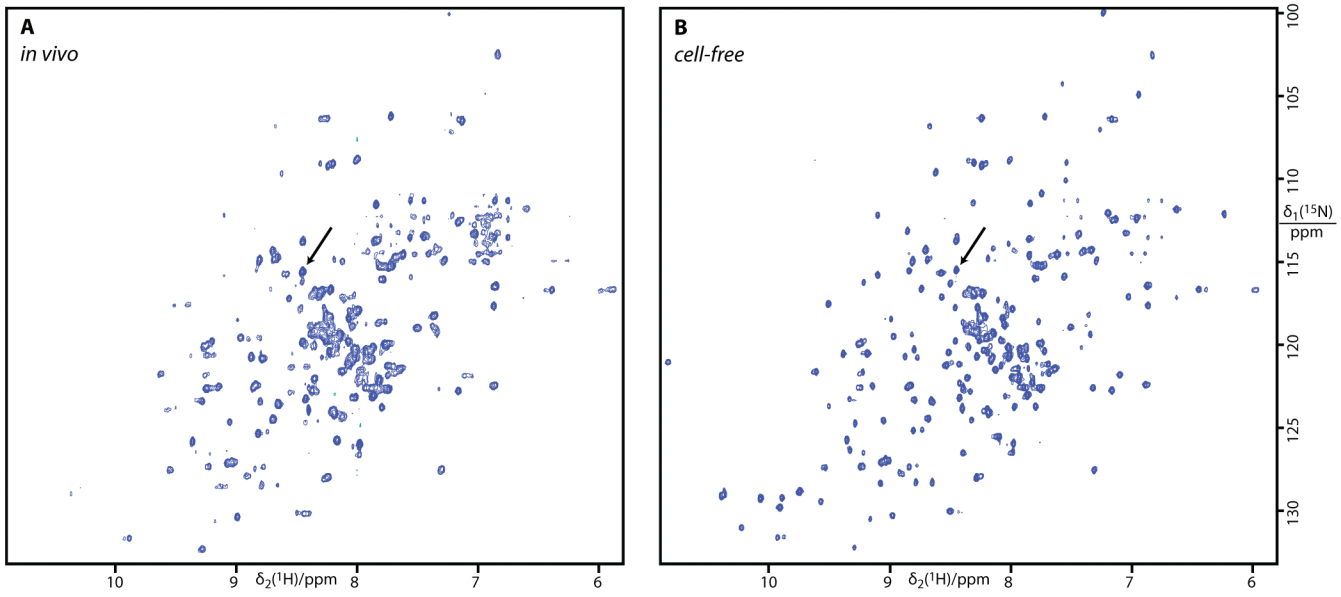

Figure 1. [^{15}N,^{1}H]-TROSY spectra of *Klebsiella pneumoniae* metallo-β-lactamase (IMP-1). The spectra were recorded at 37ºC in a buffer of 20 mM MES, pH 6.5, 100 mM NaCl, 90% H^2O/10% D_2O on a Bruker 800 MHz NMR spectrometer. The spectra were scaled to display the cross-peak of a solvent exposed residue with complete ^{2}H-^{1}H back-exchange (identified by an arrow) with the same number of contour lines in both spectra. (A) Protein sample prepared using conventional *in vivo* expression in *E. coli* in D_2O. (B) Protein sample prepared by cell-free synthesis using CIL amino acids. The spectrum was recorded in about 0.5 h. There are fewer cross-peaks in (A), mostly due to insufficient ^{2}H-^{1}H back-exchange of the amide hydrogens during purification of the sample. Complete back-exchange proved impossible even after months of storage in H_2O at 4ºC. In addition, the peaks in (A) tend to display broader line widths, which may be attributed to incomplete perdeuteration as the sample was prepared using ^{13}C-glucose instead of ^{2}H/^{13}C-glucose.

pronounced for hydrogens in the α-position and can be attributed to enzymes requiring the pyridoxal 5′-phosphate (PLP) cofactor. The cell-free synthesis method offers the crucial advantage that this ^{2}H-^{1}H exchange can be suppressed in a straightforward manner by treating the S30 extract with $NaBH_4$ to reduce PLP and its adducts with the protein.[4] This leads to irreversible inactivation of the PLP enzymes, none of which are required for protein production.

Analysis by NMR Spectroscopy

Figure 1 shows a comparison between two [^{15}N,^{1}H]-TROSY spectra of ^{2}H/^{15}N/^{13}C-labeled metallo-β-lactamase from *Klebsiella pneumonia* (IMP-1), where one sample was prepared *in vivo* in *E. coli* using a minimal medium with D_2O (Figure 1A), while the other sample was prepared by cell-free synthesis in H_2O, supplying all amino acids in ^{2}H/^{15}N/^{13}C-labeled form (Figure 1B). Clearly, more cross-peaks can be observed for the sample prepared by cell-free synthesis. IMP-1 is a metallo-enzyme that we have been unable to refold following denaturation. The protein is also prone to precipitation during NMR measurement, making prolonged incubations for ^{2}H-^{1}H back-exchange of amide hydrogens unpractical. As an added benefit of using highly perdeuterated amino acids, the cross-peaks in Figure 1B tend to be narrower than those in Figure 1A. We attribute this effect to incomplete perdeuteration in the sample prepared *in vivo*, as we used uniformly ^{13}C-labeled glucose rather than ^{2}H/^{13}C-labeled glucose.

Cost and Yield

The protein sample of Figure 1B was prepared in a 2 mL reaction mixture in an outer buffer of 20 mL. Each of the labeled CIL amino acids was supplied at a final concentration of 1 mM, *i.e.* the amino acids were supplied in total quantities ranging from 1.6 mg (glycine) to 4.7 mg (lysine•2HCl). For such small quantities of amino acids, the total cost of the amino acids from CIL ended up being significantly less than the pro-rata cost for accessing the 800 MHz NMR spectrometer to measure a 3D HNCA spectrum for backbone resonance assignment. Regarding the protein yield, purification by chromatography via an SP column, buffer exchange and concentration of the sample produced a total of 1.8 mg of purified IMP-1, corresponding to a 0.4 mM solution in a volume of 0.15 mL that was measured in a 5 mm Shigemi tube. The initial yield after synthesis was significantly higher but the protein is prone to degradation, precipitating heavily during the purification and concentration steps.

Final Remarks

Cell-free protein synthesis offers additional advantages beyond those discussed here (for a review see ref. 5). It has long been established that cell-free synthesis is the technique of choice for sample preparations in which only one or several amino acids are isotope labeled, because the amino acids are used sparingly and isotope scrambling is suppressed (see ref. 6). As only a small fraction of the amino acids is incorporated into the final protein product, further dramatic improvements in protein yield are conceivable. Unfortunately, algal hydrolysates do not produce good protein yields in our hands, so that, counter to intuition, the purchase of the 20 individual amino acids is more economical. Even so, comparing only the cost of labeled compounds needed for the production of uniformly isotope-labeled proteins, cell-free synthesis readily competes with *in vivo* preparations.

$^{2}H/^{13}C/^{15}N$-Labeled Amino Acids*

Catalog No.	Description
CDNLM-6800	L-Alanine ($^{13}C_3$, 97-99%; D_4, 97-99%; ^{15}N, 97-99%)
CDNLM-6801	L-Arginine•HCl ($^{13}C_6$, 97-99%; D_7, 97-99%; $^{15}N_4$, 97-99%)
CDNLM-6802	L-Asparagine•H_2O ($^{13}C_4$, 97-99%; D_3, 97-99%; $^{15}N_2$, 97-99%)
CDNLM-6803	L-Aspartic acid ($^{13}C_4$, 97-99%; D_3, 97-99%; ^{15}N, 97-99%)
CDNLM-6809	L-Cysteine ($^{13}C_3$, 97-99%; D_3, 97-99%; ^{15}N, 97-99%)
CDNLM-6799	Glycine ($^{13}C_2$, 97-99%; 2,2-D_2, 97-99%; ^{15}N, 97-99%)
CDNLM-6804	L-Glutamic acid ($^{13}C_5$, 97-99%; D_5, 97-99%; ^{15}N, 97-99%)
CDNLM-6805	L-Glutamine ($^{13}C_5$, 97-99%; D_5, 97-99%; $^{15}N_2$, 97-99%)
CDNLM-6806	L-Histidine•HCl•H_2O ($^{13}C_6$, 97-99%; D_5, 97-99%; $^{15}N_3$, 97-99%)
CDNLM-6807	L-Isoleucine ($^{13}C_6$, 97-99%; D_{10}, 97-99%; ^{15}N, 97-99%)
CDNLM-6808	L-Leucine ($^{13}C_6$, 97-99%; D_{10}, 97-99%; ^{15}N, 97-99%)
CDNLM-6810	L-Lysine•2HCL ($^{13}C_6$, 97-99%; D_9, 97-99%; $^{15}N_2$, 97-99%)
CDNLM-6798	L-Methionine ($^{13}C_5$, 97-99%; D_8, 97-99%; ^{15}N, 97-99%)
CDNLM-6811	L-Phenylalanine ($^{13}C_9$, 97-99%; D_8, 97-99%; ^{15}N, 97-99%)
CDNLM-6812	L-Proline ($^{13}C_5$, 97-99%; D_7, 97-99%; ^{15}N, 97-99%)
CDNLM-6813	L-Serine ($^{13}C_3$, 97-99%; D_3, 97-99%; ^{15}N, 97-99%)
CDNLM-6814	L-Threonine ($^{13}C_4$, 97-99%; D_5, 97-99%; ^{15}N, 97-99%)
CDNLM-6815	L-Tyrosine ($^{13}C_9$, 97-99%; D_7, 97-99%; ^{15}N, 97-99%)
CDNLM-6816	L-Tryptophan ($^{13}C_{11}$, 97-99%; D_8, 97-99%; $^{15}N_2$, 97-99%)
CDNLM-6817	L-Valine ($^{13}C_5$, 97-99%; D_8, 97-99%; ^{15}N, 97-99%)

$^{13}C/^{15}N$-Labeled Amino Acids*

CNLM-534-H	L-Alanine ($^{13}C_3$, 99%; ^{15}N, 99%)
CNLM-539-H	L-Arginine•HCl ($^{13}C_6$, 99%; $^{15}N_4$, 99%)
CNLM-3819-H	L-Asparagine•H_2O ($^{13}C_4$, 99%; $^{15}N_2$, 99%)
CNLM-544-H	L-Aspartic acid ($^{13}C_4$, 99%; ^{15}N, 99%)
CNLM-3871-H	L-Cysteine ($^{13}C_3$, 99%; ^{15}N, 99%)
CNLM-554-H	L-Glutamic acid ($^{13}C_5$, 99%; ^{15}N, 99%)
CNLM-1275-H	L-Glutamine ($^{13}C_5$, 99%; $^{15}N_2$, 99%)
CNLM-1673-H	Glycine ($^{13}C_2$, 99%; ^{15}N, 99%)
CNLM-758-0	L-Histidine•HCl•H_2O (<5% D) ($^{13}C_6$, 97-99%; $^{15}N_3$, 97-99%)
CNLM-561-H	L-Isoleucine ($^{13}C_6$, 99%; ^{15}N, 99%)
CNLM-281-H	L-Leucine ($^{13}C_6$, 99%; ^{15}N, 99%)
CNLM-291-H	L-Lysine•2HCl ($^{13}C_6$, 99%; $^{15}N_2$, 99%)
CNLM-759-H	L-Methionine ($^{13}C_5$, 99%; ^{15}N, 99%)
CNLM-575-H	L-Phenylalanine ($^{13}C_9$, 99%; ^{15}N, 99%)
CNLM-436-H	L-Proline ($^{13}C_5$, 99%; ^{15}N, 99%)
CNLM-474-H	L-Serine ($^{13}C_3$, 99%; ^{15}N, 99%)
CNLM-587-0	L-Threonine ($^{13}C_4$, 97-99%; ^{15}N, 97-99%)
CNLM-439-H	L-Tyrosine ($^{13}C_9$, 99%; ^{15}N, 99%)
CNLM-2475-H	L-Tryptophan ($^{13}C_{11}$, 99%; $^{15}N_2$, 99%)
CNLM-442-H	L-Valine ($^{13}C_5$, 99%; ^{15}N, 99%)

^{15}N-Labeled Amino Acids*

Catalog No.	Description
NLM-454	L-Alanine (^{15}N, 98%)
NLM-396	L-Arginine•HCl ($^{15}N_4$, 98%)
NLM-395	L-Asparagine•H_2O ($^{15}N_4$, 98%)
NLM-718	L-Aspartic acid (^{15}N, 98%)
NLM-2295	L-Cysteine (^{15}N, 98%)
NLM-202	Glycine (^{15}N, 98%)
NLM-135	L-Glutamic acid (^{15}N, 98%)
NLM-1328	L-Glutamine ($^{15}N_2$, 98%)
NLM-1513	L-Histidine•HCl•H_2O ($^{15}N_3$, 98%)
NLM-292	L-Isoleucine (^{15}N, 98%)
NLM-142	L-Leucine (^{15}N, 98%)
NLM-1554	L-Lysine•2HCl ($^{15}N_2$, 98%)
NLM-752	L-Methionine (^{15}N, 98%)
NLM-108	L-Phenylalanine (^{15}N, 98%)
NLM-835	L-Proline (^{15}N, 98%)
NLM-2036	L-Serine (^{15}N, 98%)
NLM-742	L-Threonine (^{15}N, 98%)
NLM-590	L-Tyrosine (^{15}N, 98%)
NLM-800	L-Tryptophan ($^{15}N_2$, 98%)
NLM-316	L-Valine (^{15}N, 98%)

Selective $^{2}H/^{13}C/^{15}N$-Labeled Amino Acids*

CDNLM-4279	L-Alanine ($^{13}C_3$, 95-97%; ^{15}N, 96-99%; 2-D, 97%+)
CDLM-8649	L-Alanine (3-^{13}C, 99%; 2-D, 96%)
CDNLM-4282	L-Isoleucine ($^{13}C_6$, 95-97%; ^{15}N, 96-99%; 2,3-D_2, 97%+)
CDNLM-4280	L-Leucine ($^{13}C_6$, 95-97%; ^{15}N, 96-99%; 2,3,3-D_3, 97%+)
CDLM-8885	L-Methionine (2,3,3,4,4-D_5, 98%; methyl-$^{13}CH_3$, 99%)
CDNLM-4281	L-Valine ($^{13}C_5$, 95-97%; ^{15}N, 96-99%; 2,3-D_2, 97%+)
CNLM-7610-0	L-Tyrosine (2,3-$^{13}C_2$, 99%; ^{15}N, 98%)
CNLM-7611-PK	L-Phenylalanine (2,3-$^{13}C_2$, 99%; ^{15}N, 98%)

Mixes for Cell-Free Synthesis

CDNLM-6784	"Cell Free" amino acid mix (20 AA) (U-^{13}C, 97-99%; U-^{15}N, 97-99%; U-D, 97-99%)
CNLM-6696	"Cell Free" amino acid mix (20 AA) (U-^{13}C, 97-99%; U-^{15}N, 97-99%)
NLM-6695	"Cell Free" amino acid mix (20 AA) (U-^{15}N, 97-99%)

***25 mg and 50 mg packaged sizes are available.**

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