Supporting Information for "Quantitation of Recombinant Protein in Whole Cells and Cell Extracts by Solid-State NMR Spectroscopy" by E. P. Vogel and D. P Weliky

A. RP plasmid and cell types

Table SI1. RP plasmids and *E. coli* cell types

Sample type	Recombinant protein (RP)	Plasmid type	Cell type
RP^-	None	pET24a+	Rosetta2
RP^+	Fgp41	pET24a+	Rosetta2
RP^+	Fgp41+	pET24a+	Rosetta2
RP^+	FHA2	pET24a+	Rosetta2
RP^+	Hairpin	pGEMT	BL21(DE3)
RP^+	Human Proinsulin (HPI)	pQE-31	BL21(DE3)

Table SI2. RP amino acid sequences[a]

Fgp41

A V G L G A V F L G F L G A A G S T M G A A S M T L T V Q A R Q L L S G I V Q Q Q S N L L K A I E A Q Q H L L K L T V W G I K Q L Q A R V L A V E R Y L Q D Q Q L L G I W G A S G K L I A T S F V P W N N S W S N K T Y N E I W D N M T W L Q W D K E I S N Y T D T I Y R L L E D S Q N Q Q E K N E Q D L L A L D K L E H H H H H H

Fgp41+

A V G L G A V F L G F L G A A G S T M G A A S M T L T V Q A R Q L L S G I V H Q Q S N L L K A I E A Q Q H L L K L T V W G I K Q L Q A R V L A V E R Y L Q D Q Q L L G I W G A S G K L I A T S F V P W N N S W S N K T Y N E I W D N M T W L Q W D K E I S N Y T D T I Y R L L E D S Q N Q Q E K N E Q D L L A L D K W A N L W N W F S I T N W L W Y I K L E H H H H H H

FHA2

G L F G A I A G F I E N G W E G M I D G W Y G F R H Q N S E G T G Q A A D L K S T Q A A I D Q I N G K L N R V I E K T N E K F H Q I E K E F S E V E G R I Q D L E K Y V E D T K I D L W S Y N A E L L V A L E N Q H T I D L T D S E M N K L F E K T R R Q L R E N A E E M G N G S F K I Y H K A D N A A I E S I R N G T Y D H D V Y R D E A L N N R F Q I K G V E L K S G Y K D W V E H H H H H H

Hairpin

C T L T V Q A R Q L L S G I V Q Q Q N N L L R A I E A Q Q H L L Q L T V W G I K Q L Q A R I L S G G R G G W M E W D R E I N N Y T S L I H S L I E E S Q N Q Q E K N E Q E L L E L D K W

Human Proinsulin (HPI)

G S S H H H H H H S S G L D P V L M F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K T R R E A E D L Q V G Q V E L G G G P G A G S L Q P L A L E G S L Q K R G I V E Q C C T S I C S L Y Q L E N Y C N

[a] Underlined regions contain non-native residues that were inserted into the sequence.

B. Materials

The Fgp41 plasmid included an insert corresponding to the 154 N-terminal residues of the gp41 protein of HIV-1 (Q45D5 primary isolate). The human proinsulin (HPI) plasmid was obtained from Dr. Robert B. Mackin at Creighton University and contained an insert corresponding to the 87 residues of human proinsulin. The FHA2 plasmid was obtained from Dr. Yeon-Kyun Shin at Iowa State University and contained an insert corresponding to the 185 N-terminal residues of the HA2 subunit of the hemagglutinin protein of the influenza virus (X31 strain). The Hairpin plasmid was obtained from Dr. Kelly Sackett at Michigan State University and contained an insert corresponding to residues 23-70 and 117-145 of the gp41 protein of HIV-1 (HXB2 laboratory strain). The insert also included a SGGRGG linker between the two gp41 regions. Each plasmid was transformed into either BL21(DE3) or BL21(DE3) Rosetta2 chemically competent *E. coli* cells (Novagen, Gibbstown, NJ). Other reagents and their sources include: Luria-Bertani Broth (LB) medium (Acumedia, Lansing, MI); isopropyl-β-D-thiogalactopyranoside (IPTG) (Anatrace, Maumee, OH); isotopically labeled amino acids (Cambridge Isotope Laboratories, Andover, MA). Other materials were obtained from Sigma-Aldrich (St. Louis, MO).

C. Sample preparation

One 250 mL flask containing 100 mL of LB and the proper antibiotic was inoculated with 0.5 mL of a glycerol stock of *E. coli* cells that contained a specific plasmid. The flask was placed in an incubator shaker with shaking at 180 rpm and a temperature of 37 °C. After 16 hours, the OD_{600} was ~4 and the cells were harvested by centrifugation at 10,000*g* at 4°C for 10 minutes. The cells were then resuspended into a 250 mL baffled flask containing 50 mL of M9 minimal medium, antibiotic, 100 µL of 1.0 M $MgSO_4$, and 250 µL of 50% v/v glycerol. After about one hour of shaking at 180 rpm at 37 °C, log(OD_{600}) vs time was linear and the *E.* coli cells were induced by addition of IPTG to a concentration of 2.0 mM. Induction continued for 3 hours with shaking at 37 °C. For many growths, a dry mixture was prepared that contained 10 mg of each of the 20 common amino acids. One or two of the amino acids typically also contained ^{13}CO and/or α-^{15}N isotopic labeling. This mixture was added to the medium just before addition of IPTG and an equivalent mixture was added after one hour of induction. After three hours of expression, the cells were harvested by centrifugation at 10,000 *g* at 4°C for 10 minutes. The cell pellet (~0.5 g wet mass) was stored at -20 °C.

"Whole cell" (WC) NMR samples were lyophilized cell pellets. "Insoluble cell pellet" (ICP) samples were prepared by first suspending ~0.5 g whole cell pellet in ~40 mL PBS (pH 7.3) followed by placement on ice. Lysis of the *E. coli* cells was achieved by sonication with a tip sonifier (4 one minute cycles, 80% amplitude, 0.8 seconds on, 0.2 seconds off). After sonication, the suspension was centrifuged at 50,000*g* at 4°C for 20 minutes. The supernatant (containing soluble proteins) was discarded and the ICP with inclusion body (IB) RP was not lyophilized.

D. SDS-PAGE

10 mg of ICP was suspended in 100 µL of SDS-containing sample buffer. Each suspension was vortexed, boiled for 5 minutes, and then centrifuged for one minute. The supernatant was diluted 5X with SDS-containing sample buffer and 15 µL of this solution was added to a well in the electrophoretic apparatus.

E. Solid-state NMR (SSNMR)

The sample (either WC or ICP) was packed into a 4 mm diameter solid-state NMR magic angle spinning (MAS) rotor. The active sample volume of the rotor was ~40 µL. Data were obtained with a 9.4 T instrument (Agilent Infinity Plus) and a triple-resonance MAS probe whose rotor was cooled with nitrogen gas at –20 °C. Experimental parameters included: (1) 8.0 kHz MAS frequency; (2) 5 µs ^{1}H π/2 pulse and 2 ms cross-polarization time with 50 kHz ^{1}H field and 70-80 kHz ramped ^{13}C field; (3) 1 ms rotational-echo double-resonance (REDOR) dephasing time with a 9 µs ^{13}C π pulse at the end of each rotor period except the last period and for the S_1 acquisition, a 12 µs ^{15}N π pulse at the center of each rotor period; and (4) ^{13}C detection with 90 kHz two-pulse phase modulation ^{1}H decoupling (which was also on during the dephasing time); and (5) 0.8 sec pulse delay. Data were acquired without (S_0) and with (S_1) ^{15}N π pulses during the dephasing time and respectively represented the full ^{13}C signal and the signal of ^{13}Cs not directly bonded to ^{15}N nuclei.[1,2] Spectra were externally referenced to the methylene carbon of adamantane at 40.5 ppm so that the ^{13}CO shifts could be directly compared to those of soluble proteins.[3]

F. Suppression of scrambling of isotopic labels

Experimental design of the HCN variant of the method included targeting a specific dipeptide sequence in the RP through addition to the expression medium of either: (1) one ^{13}CO-labeled amino acid and one ^{15}N-labeled amino acid; or (2) one single ^{13}CO,^{15}N-labeled amino acid. Glycerol was the only other carbon source and NH_4Cl was the only other nitrogen source in the expression medium. A negative control "RP^{+}_{lab}" whole cell (WC) sample was prepared with this protocol using cells that contained the FHA2 plasmid and with ^{13}CO-Ala and ^{15}N-Val in the expression medium. As shown in **Fig. SI1a**, some ΔS signal was observed even though there are no AV dipeptides in the FHA2 sequence. This signal may be due to AV dipeptides in other proteins produced during the expression period. Alternatively, there could be metabolic scrambling in the bacteria of the ^{13}CO label into other "X" amino acids and/or scrambling of the ^{15}N label into other "Y" amino acids with consequent ΔS signal from AY, XV, or XY dipeptides in the FHA2 sequence. The protocol was modified to suppress scrambling by first preparing a solid mixture containing 10 mg each of: (1) ^{13}CO-Ala; (2) ^{15}N-Val; and (3) each of the 18 other amino acids (unlabeled). This mixture was added to the culture just prior to induction and an equivalent mixture was added after one hour of induction. Scrambling was greatly reduced with the modified protocol as evidenced by a ΔS signal (**Fig. SI1b**) that was ~3-times smaller than the signal of the sample prepared with the initial protocol (**Fig. SI1a**). The suppression of scrambling was likely through product feedback inhibitory loops of the bacterial amino acid metabolic pathways.[4]

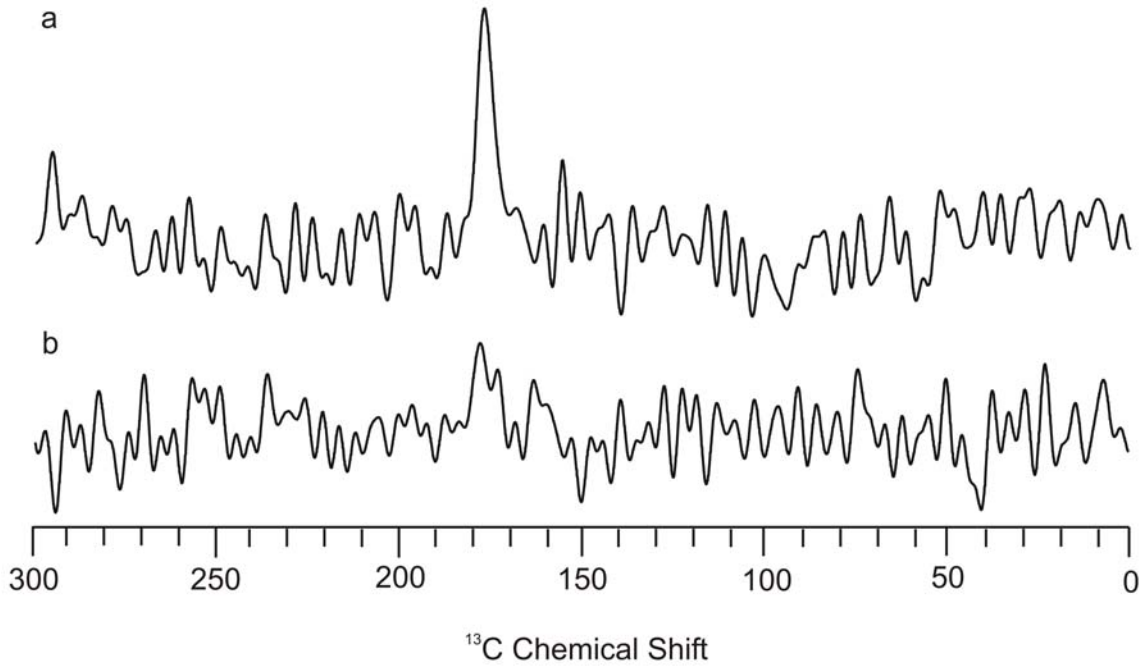

Figure SI1: Δ*S* spectra for WC samples prepared using expression medium that contained: (a) ^{13}CO-Ala and ^{15}N-Val as well as glycerol and NH_4Cl; or (b) ^{13}CO-Ala, ^{15}N-Val, and the other 18 amino acids in unlabeled form, as well as glycerol and NH_4Cl. Each Δ*S* spectrum was the result of the difference between (a) 46652 S_0 and 46652 S_1 scans or (b) 43647 S_0 and 43647 S_1 scans. There is no line broadening or baseline correction. The panel a integrated intensity in the ^{13}CO chemical shift range (185-170 ppm) was 58.1 $\pm$ 9.9 and the panel b intensity was 21.3 $\pm$ 8.3. Each uncertainty was the standard deviation of the integrated intensities in 13 other regions of the spectrum for which no ΔS intensity is expected, cf. **Table SI3** below. The difference in the standard deviations of the two samples was not statistically significant at the 95% confidence level as evaluated by the F test. For the 12 degrees of freedom in each data set, the critical value of F is 2.69 which is greater than the 1.43 value calculated from the data.

Table SI3. Integrated intensities of the **Fig. SI1** spectra[a]

Shift integration interval (ppm)	Spectrum a	Spectrum b
185 → 170 ≡ $I_{\Delta S}$	58.1279	21.341
400 → 385	2.6401	5.7787
380 → 365	-14.3034	5.7584
360 → 345	-0.0376	-22.852
340 → 325	-4.2526	-3.7395
320 → 305	-6.1709	2.6227
300 → 285	17.543	-17.4505
280 → 265	-8.8076	-12.6827
240 → 225	-12.7001	9.506
220 → 205	-8.9171	-10.7618
0 → -15	-2.5523	-7.8094
-20 → -35	-2.0299	2.4773
-40 → -55	4.2796	2.4773
-60 → -75	-0.3178	-1.3408

[a] The $I_{\Delta S}$ is the integrated intensity in the 185 → 170 ppm interval. No significant signal is expected in the other intervals and the uncertainty in $I_{\Delta S}$ is the standard deviation of the integrated intensities in these intervals.

G. Criterion for quantitative labeling of the RP

This calculation shows the 20 mg aliquot of labeled amino acid in the 50 mL expression medium is sufficient for quantitative labeling of a RP. Consider that RP ≡ Hairpin, the labeled amino acid ≡ Leu, and the expression level = 500 mg Hairpin/L culture. There are 14 Leu's in the 92-residue Hairpin and mass Leu/mass Hairpin = 0.15. This is a "worst-case" example for labeling because of the high Leu mass fraction in Hairpin and because of the high expression level (which is two times greater than the already-high experimentally observed Hairpin expression level).

Minimum required mg Leu in 50 mL culture =

$[5 \times 10^2$ mg Hairpin/L$] \times [5 \times 10^{-2}$ L$] \times [$(mole Hairpin)$/(1.0723 \times 10^7$ mg Hairpin$)]$

$\times$ [(14 mole Leu)/(mole Hairpin)] $\times$ $[(1.33 \times 10^5$ mg Leu)/(mole Leu)]

= 4 mg Leu

H. Standard Curve Experiments

Three samples were prepared. For the first sample, 25 mg ^{13}CO,^{15}N-Leu was massed and then manually mixed with sufficient talc to fill the ~40 μL volume of the rotor. The second sample was similarly prepared but with 5 mg ^{13}CO,^{15}N-Leu. For the third sample, 1 mg of ^{13}CO,^{15}N-Leu was manually mixed with sufficient talc to fill two rotor volumes and half of this mixture was used for the experiment. **Fig. SI2** displays the ^{13}CO spectra of these three samples, **Table SI4** displays the I_{CO} values determined from the spectral intensities, and **Fig. SI3** displays a plot of the I_{CO} vs mole ^{13}CO as well as best-fit line.

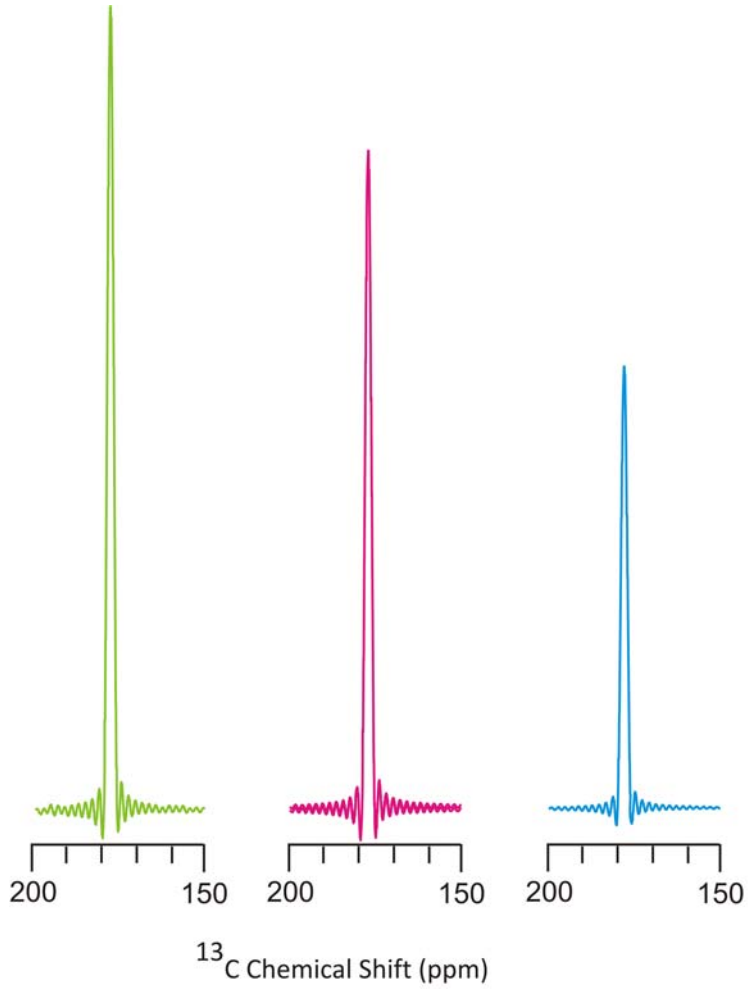

Figure SI2. ^{13}C spectra of samples containing ^{13}CO,^{15}N-Leu mixed with talc. The samples for the green, pink, and blue spectra respectively contained 0.5, 5, and 25 mg of ^{13}CO,^{15}N-Leu. The relative scaling factors of the green, pink and blue spectra are 50, 5, and 1 and were chosen to facilitate assessment of the degree of linearity of intensity vs mass ^{13}CO,^{15}N-Leu. Each REDOR S_0 spectrum was acquired with 1 ms dephasing time and is the sum of 50,000 scans. Each spectrum was processed with 200 Hz Gaussian line broadening and 5th order polynomial baseline correction.

Table SI4. I_{CO} values for the $^{13}CO,^{15}N$-Leu/talc samples

$^{13}CO,^{15}N$-Leu (mg)	$^{13}CO,^{15}N$-Leu (moles)	I_{CO}[a]
0.5	3.75×10^{-6}	1432 (12)
5	3.75×10^{-5}	11666 (12)
25	1.88×10^{-4}	40603 (12)

[a] The uncertainty was calculated based on the spectral noise of the sample containing 0.5 mg $^{13}CO,^{15}N$-Leu. This spectrum was chosen because it had the smallest fluctuations arising from truncation of the free-induction data. The intrinsic (non-truncation) noise should be the same for all spectra because each was the sum of the same number of scans on the same spectrometer.

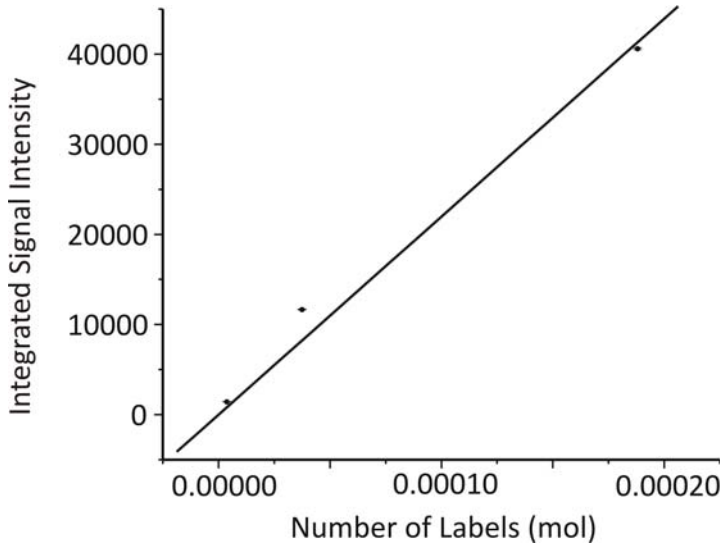

Figure SI3. I_{CO} vs mole ^{13}CO for three different samples prepared with $^{13}CO,^{15}N$-Leu manually mixed with talc to fill the 4 mm rotor volume. The best $y = mx$ linear fit to the data is $I_{CO} = 2.12(13) \times 10^8$ mole ^{13}CO. The best-fit $R^2 = 0.9852$.

I. HC determination of RP expression

Fig. 1a displays the HC variant ^{13}C spectra of the RP^{-}_{lab} and most RP^{+}_{lab} ICP samples and **Fig. SI4** displays additional ^{13}C spectra of the HPI samples.

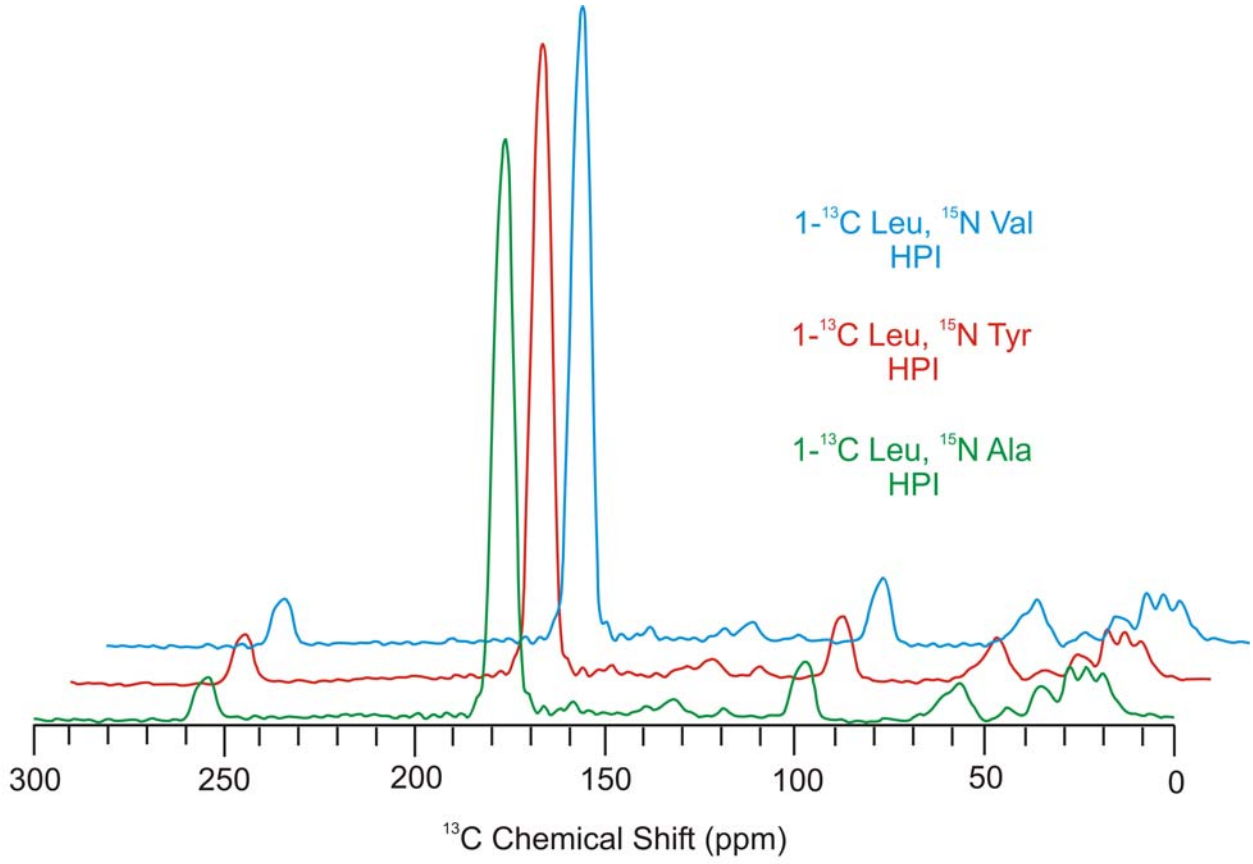

Figure SI4. ^{13}C REDOR S_0 spectra with 1 ms dephasing time for three different ICP RP^{+}_{lab} samples all with RP = HPI and ^{13}CO-Leu labeling. The ^{15}N labeling differed among the samples but ^{15}N nuclei do not affect the S_0 spectrum. Each spectrum is the sum of 50,000 scans and was processed with 100 Hz Gaussian line broadening and a 5th order baseline correction. Minor scaling of the intensities was done to have more equal aliphatic (0-90 ppm) intensities among the three spectra.

The RP expression level is calculated with **Eq. SI1** and the underlying rationales for this equation and descriptions of the terms are presented below.

Mass RP/volume culture =

$$\{(I_{CO}^{+}/I_{Al}^{+}) - (I_{CO}^{-}/I_{Al}^{-})\}/V_c \times I_{Al}^{0} \times C \times \mathrm{MW}_{RP}/N_{lab} \qquad \text{(SI1)}$$

For spectra of both the RP^{+}_{lab} and RP^{-}_{lab} ICP samples, integrated intensities (I) were measured for both the $^{13}CO \equiv$ 170-185 ppm region (ie. I_{CO}^{+} and I_{CO}^{-}) and the aliphatic (Al) $\equiv$ 0-90 ppm region (ie. I_{Al}^{+} and I_{Al}^{-}). The I_{CO} and I_{Al} for all samples are presented in **Table SI5**. There are large variations among the I_{CO} values with the smallest value for the RP^{-}_{lab} sample, as expected.

The (I_{CO}^{+}–$I_{CO}^{-}{}_{lab}$) difference should be proportional to the number of moles of ^{13}CO-Leu in the RP in the NMR sample. However, there is sample-to-sample variation in the total ICP mass packed into the NMR rotor and this should be accounted for because mass RP in the rotor is proportional to the mass ICP in the rotor. We considered that the ICP mass $\propto I_{Al}$ as justified by: (1) the aliphatic ^{13}C signal should not be affected by incorporation of ^{13}CO-Leu into protein produced during the expression period; and (2) the **Table SI5** data don't show any clear correlation between the I_{CO} and I_{Al} values of a sample; ie. I_{Al} is approximately independent of RP quantity. The effect of the RP^{+}_{lab} sample mass was accounted for with multiplication by I_{Al}^{0}/I_{Al}^{+} where I_{Al}^{0} corresponds to a "typical" sample. The effect of the RP^{-}_{lab} sample mass was accounted for by a similar factor I_{Al}^{0}/I_{Al}^{-}. For numerical calculation of RP expression levels, I_{Al}^{0} = 1000 was chosen as the "typical" value, cf. **Table SI5**.

C is the standard curve-derived coefficient (mole ^{13}CO)/I_{CO} ratio that is specific to the SSNMR spectrometer and probe. For the present study, $C = 4.72\times10^{-9}$ mole ^{13}CO and was determined using the standard curve and analysis presented in Section H.

V_c corresponds to the culture volume needed to prepare an ICP that fills the NMR rotor. V_c = 25 mL was used for the numerical calculations because about two rotors could be filled with the ICP from a 50 mL culture.

The (moles ^{13}CO-Leu in the RP)/(volume culture) was converted to (mass RP)/(volume culture) with multiplication by MW_{RP}/N_{lab} where $\mathrm{MW}_{RP} \equiv$ RP molecular weight and $N_{lab} \equiv$ number of labeled residues in the RP. The expression levels calculated with **Eq. SI1** are presented in **Table SI6**.

The calculations assume quantitative ^{13}CO-Leu labeling of the RP. For f_{CO} fractional labeling, we expect that (I_{CO}^{+}/I_{Al}^{+})–(I_{CO}^{-}/I_{Al}^{-}) will be reduced by a factor of ~f_{CO}. The **Table SI6** calculated levels are therefore likely lower limits on RP expression.

Table SI5. ^{13}C signal intensities of the ^{13}CO-Leu labeled ICP samples [a,b]

Sample type	RP	Labeled amino acid(s)	I_{CO}	I_{Al}	I_{Al}^0/I_{Al}
RP^-_{lab}	none	^{13}CO,^{15}N-Leu	353	802	1.25
RP^+_{lab}	Fgp41	^{13}CO,^{15}N-Leu	1041	926	1.08
RP^+_{lab}	Fgp41+	^{13}CO,^{15}N-Leu	918	857	1.17
RP^+_{lab}	FHA2	^{13}CO,^{15}N-Leu	931	667	1.50
RP^+_{lab}	Hairpin	^{13}CO,^{15}N-Leu	1269	576	1.74
RP^+_{lab}	HPI	^{13}CO-Leu + ^{15}N-Val	3708	1338	0.75
RP^+_{lab}	HPI	^{13}CO-Leu + ^{15}N-Ala	2934	931	1.07
RP^+_{lab}	HPI	^{13}CO-Leu + ^{15}N-Tyr	3796	1150	0.87

[a] Integration windows are 170-185 ppm for I_{CO} and 0-90 ppm for I_{Al}.
[b] I_{Al}^0 is for a typical sample and was set to 1000.

Table SI6. RP expression calculated using the HC approach [a]

RP	Labeled amino acid(s)	$I_{Al}^0 \times [(I_{CO}^+/I_{Al}^+) - (I_{CO}^-/I_{Al}^-)]$	N_{lab}	MW_{RP}(Da)	Expression (mg RP/L culture)	Expression (μmol RP/L culture)
Fgp41	^{13}CO,^{15}N-Leu	684	24	18376	105 (1)	5.7 (1)
Fgp41+	^{13}CO,^{15}N-Leu	632	26	20809	101 (2)	4.9 (1)
FHA2	^{13}CO,^{15}N-Leu	956	13	22363	329 (4)	14.7 (2)
Hairpin	^{13}CO,^{15}N-Leu	1763	14	10723	270 (2)	25.2 (2)
HPI	^{3}CO-Leu + ^{15}N-Val	2331	14	11348	378 (3)	33.3 (2)
HPI	^{13}CO-Leu + ^{15}N-Ala	2710	14	11348	439 (3)	38.7 (2)
HPI	^{13}CO-Leu + ^{15}N-Tyr	2862	14	11348	464 (3)	40.9 (2)

[a] The uncertainties in expression levels are calculated from spectral noise. The expression levels for the three samples with RP = HPI indicate that the typical sample-to-sample variation in expression is ±10%.

J. HCN determination of RP expression

Fig. SI5 displays the S_0 and S_1 spectra associated with **Fig. 2**, **Table SI7** lists the parameters of the best-fit deconvolutions of the **Fig. 2** ΔS spectra, **Fig. SI6** displays the ΔS spectra for the RP^-_{lab} and the RP^+_{lab} samples, and **Table SI8** lists the peak shifts, line widths, and $I_{\Delta S}$ for the RP^+_{lab} spectra. These figures and tables are followed by a description of the HCN approach to determination of RP expression.

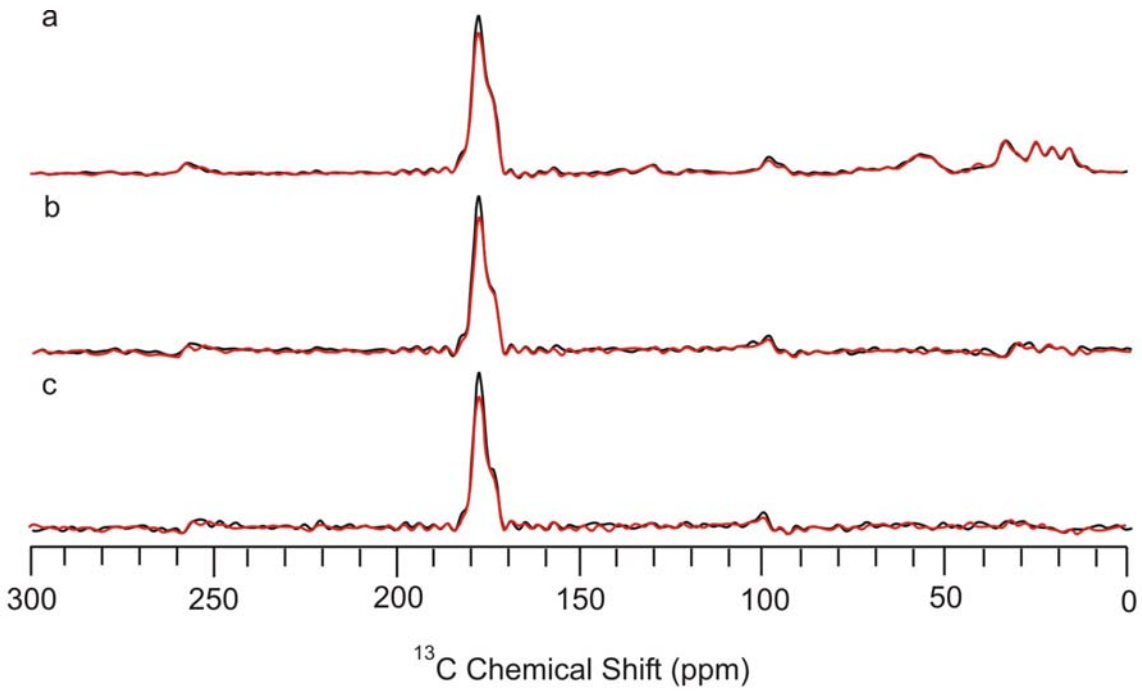

Figure SI5: S_0 (black) and S_1 (red) spectra used for the ΔS spectra displayed in **Fig. 2** in the main text. For panel a, the spectra are from a single RP^+_{lab} ICP sample for which the cells contained the Fgp41 plasmid and the expression medium contained ^{13}CO,^{15}N-Leu. For panel b, the displayed spectra are differences between the (RP^+_{lab}) and (RP^-_{lab}) samples, ie. $S_0(RP^+_{lab})–S_0(RP^-_{lab})$ and $S_1(RP^+_{lab})–S_1(RP^-_{lab})$. For panel c, the displayed spectra are differences between the (RP^+_{lab}) and (RP^+_{na}) samples, ie. $S_0(RP^+_{lab})–S_0(RP^+_{na})$ and $S_1(RP^+_{lab})–S_1(RP^+_{na})$. The same vertical scale was used for all spectra. Spectral processing included 5th order polynomial baseline correction and no line broadening. Each of the experimental spectra was the sum of 50,000 scans.

Table SI7. Best-fit deconvolutions of Fig. 2 ΔS spectra [a]

Fig. 2 spectrum	Peak ^{13}C shift (ppm)	Linewidth (ppm)	Integrated intensity
a	178.4	3.0	61 (7)
b	178.4	3.1	57 (6)
c	178.4	2.8	53 (5)

[a] The parameters are for the best-fit Gaussian lineshape of the dominant ^{13}CO spectral peak including full-width at half-maximum linewidth. The uncertainty in integrated intensity is the RMSD spectral noise in a 5 ppm region.

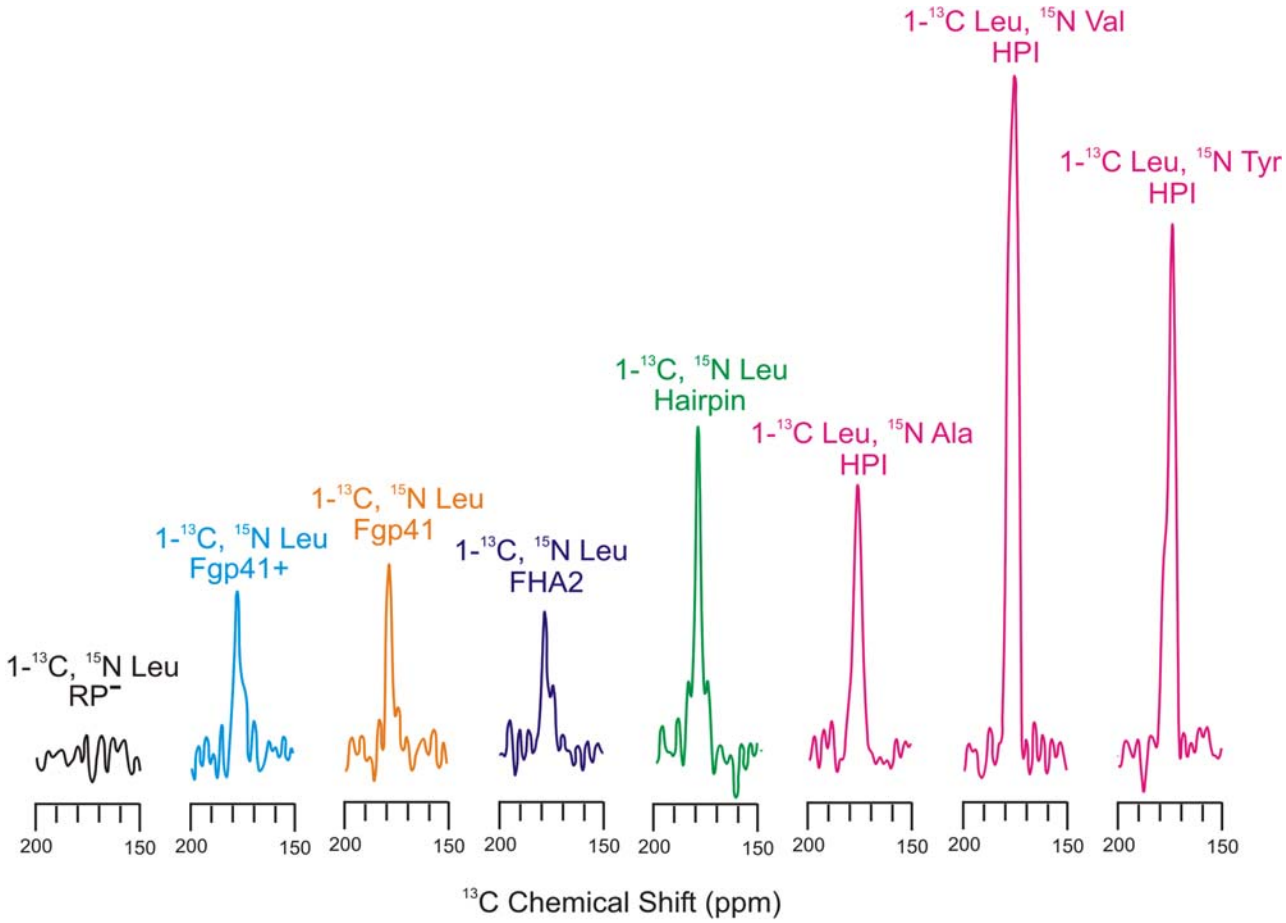

Figure SI6. ΔS spectra of the ^{13}CO region of the RP^-_{lab} and RP^+_{lab} ICP samples with the indicated ^{13}CO and ^{15}N labeling and RP. Each ΔS spectrum was the difference between 50,000 S_0 and 50,000 S_1 scans and was processed without line broadening and with 5th order polynomial baseline correction. The spectra are displayed with the same vertical scale.

Table SI8. Analysis of the ΔS spectra of the RP^{+}_{lab} ICP samples

RP	Labeled amino acid(s)	Peak shift (ppm)	Linewidth (ppm) [a]	$I_{\Delta S}$ [b]
Fgp41[b]	^{13}CO,^{15}N-Leu	178.3	3.5	79
Fgp41+	^{13}CO,^{15}N-Leu	178.1	4.5	79
FHA2	^{13}CO,^{15}N-Leu	178.6	3.4	71
Hairpin	^{13}CO,^{15}N-Leu	178.6	3.4	139
HPI	^{13}CO-Leu + ^{15}N-Val	175.2	7.0	395
HPI	^{13}CO-Leu + ^{15}N-Ala	176.6	5.3	146
HPI	^{13}CO-Leu + ^{15}N-Tyr	174.3	4.7	251

[a] Linewidth is full-width at half-maximum of the isotropic ^{13}CO peak.
[b] Each $I_{\Delta S}$ was determined from integration over the isotropic ^{13}CO spectral region. The typical RMSD spectral noise was ~5 as determined from integrations over other regions of the ΔS spectrum.

Table SI8 presents the $I_{\Delta S}$ integrated ^{13}CO intensities for all RP^{+}_{lab} ICP samples. The $I_{\Delta S} \approx 0$ for the RP^{-}_{lab} sample, cf. **Fig. SI6**. HCN RP expression was calculated using **Eq. SI2** and the underlying rationales for this equation as well as descriptions of terms are presented below.

$$\text{Mass RP/volume culture} = I_{\Delta S}/V_c \times C/0.7 \times MW_{RP}/N_{dip} \quad \text{(SI2)}$$

The $I_{\Delta S}/V_c \times C/0.7$ = (mole ^{13}CO-Leu in labeled ^{13}CO-^{15}N dipeptides in the RP in the rotor)/(volume culture). These ICP samples were prepared from V_c = 25 mL culture volume. The $C = 4.72 \times 10^{-9}$ mole ^{13}CO is the experimentally-determined conversion factor of spectral intensity to mole ^{13}CO, cf. section H. The 0.7 factor is the fractional dephasing of a directly bonded ^{13}CO-^{15}N spin pair with 1 ms REDOR dephasing time.[5,6] The (mole ^{13}CO-Leu in labeled ^{13}CO-^{15}N dipeptides in the RP)/(volume culture) is converted to (mass RP)/(volume culture) by multiplication by MW_{RP}/N_{dip} where $MW_{RP} \equiv$ RP molecular weight and $N_{dip} \equiv$ number of labeled dipeptides in the RP. **Table SI9** presents the $I_{\Delta S}$, N_{dip}, MW_{RP}, and calculated RP expression levels.

Eq. SI2 assumes quantitative ^{13}CO and ^{15}N labeling of the RP. For f_{CO} and f_N fractional labelings, $I_{\Delta S}$ will be reduced by a factor of ~($f_{CO} \times f_N$) so the **Table SI9** calculated levels are likely lower limits on RP expression.

Table SI9. HCN determination of RP expression [a]

RP	Labeled amino acid(s)	$I_{\Delta S}$	N_{dip}	MW_{RP} (Da)	Expression (mg RP/L culture)	Expression (μmol RP/L culture)
Fgp41	^{13}CO,^{15}N-Leu	78.6	6	18376	65 (4)	3.5 (2)
Fgp41+	^{13}CO,^{15}N-Leu	78.9	6	20809	74 (4)	3.5 (2)
FHA2	^{13}CO,^{15}N-Leu	71.1	1	22363	429 (23)	19.2 (1.0)
Hairpin	^{13}CO,^{15}N-Leu	138.6	4	10723	100 (4)	9.3 (4)
HPI	^{13}CO-Leu + ^{15}N-Val	394.6	2	11348	603 (7)	53.2 (6)
HPI	^{13}CO-Leu + ^{15}N-Ala	146.3	1	11348	447 (17)	39.4 (1.5)
HPI	^{13}CO-Leu + ^{15}N-Tyr	250.8	2	11348	384 (4)	33.8 (4)

[a] Uncertainties in expression levels are based on spectral noise.

Unlike the HC approach, determination of RP expression with the HCN approach did not take into account the ICP mass that was packed into the NMR rotor. This reflects the possibility that the HCN approach could be applied to a single RP^{+}_{lab} sample. For this case, it would be difficult to assess the "typical" value I_{Al}^{0} and the I_{Al}^{0}/I_{Al}^{+} scaling factor. For the present study, we have I_{Al} values from several RP^{+}_{lab} and RP^{-}_{lab} samples and have estimated I_{Al}^{0} = 1000. Inclusion of the I_{Al}^{0}/I_{Al} scaling factor in the HCN analysis improves agreement between the expression levels calculated with the HC and HCN approaches. In particular, the HCN expression level for Hairpin increases from 100 to 175 mg/L and becomes much closer to the 270 mg/L level calculated with the HC approach.

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