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NMR-Based Screening of Proteins Containing ^{13}C-Labeled Methyl Groups

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Abstract: A method is described for NMR-based screening that involves monitoring the ^{13}C/^{1}H chemical shift changes of a protein selectively labeled with ^{13}C at the methyl groups of valine, leucine, and isoleucine (δ1 only). Using this approach, the sensitivity is increased by nearly 3-fold compared with that of NMR-based screening using ^{1}H/^{15}N chemical shifts. A synthetic route is described for the inexpensive production of the labeled amino acid precursors [3,3′-^{13}C]-α-ketoisovalerate and [3-^{13}C]-α-ketobutyrate, making the cost of protein preparation comparable to that of uniform ^{15}N labeling. In addition to enhancing the NMR-based screening efforts directed against low molecular weight proteins (MW ≤ 30 kDa), the use of the selective methyl labels in combination with deuterium labeling is advantageous for screening high molecular weight protein targets (MW ≥ 100 kDa).

Introduction

NMR-based screening is a valuable tool for lead identification and optimization in the drug discovery process. In particular, NMR screens which utilize perturbations in ^{15}N/^{1}H amide chemical shift changes of a target protein upon ligand binding have led to the discovery of novel lead compounds for a variety of therapeutic targets.[1−3] The success of this chemical-shift-based screening method is due in large part to the simplicity of the assay system (composed of only the target and test compounds), the elimination of background signals via spectral editing, and the ability to discriminate between different binding sites on the protein surface. However, the method is generally restricted to small proteins (MW ≤ 30 kDa) and the screening rate is limited by the low sensitivity of the NMR experiment. One approach to increase sensitivity is to use cryogenic probe technology, in which gains in signal-to-noise of more than 2−3 fold and screening rates of 10 000 compounds/day have been reported.[4] In principle, the sensitivity of heteronuclear NMR-based screening experiments could also be increased by observing the three protons attached to ^{13}C-labeled methyl groups as opposed to the single proton of ^{15}N-labeled amide groups. This approach may also extend the utility of NMR-based screening to larger systems, due to the favorable relaxation properties of methyl groups. However, the substantial cost of ^{13}C labeling using ^{13}C-glucose as the carbon source typically prohibits the generation of the amount of protein needed for NMR-based screening. In addition, the presence of ^{13}C−^{13}C coupling can reduce the potential 3-fold gain in signal to noise.

Here we describe a cost-effective ^{1}H/^{13}C NMR screening strategy that significantly increases the sensitivity of the NMR assay and can be used to screen proteins with molecular weights which exceed 40 kDa. The method involves selective ^{13}C labeling of the methyl groups of valine, leucine, and isoleucine (δ1 only) residues in the protein target. Ligand binding is detected by the observation of chemical shift changes in ^{1}H/^{13}C-HSQC spectra of the protein upon addition of the test compound. To achieve this, we developed an efficient synthetic protocol for the inexpensive production of the labeled amino acid precursors [3,3′-^{13}C]-α-ketoisovalerate and [3-^{13}C]-α-ketobutyrate, which are biosynthetically incorporated into the target protein when expressed in bacterial systems. The utility of this strategy is demonstrated on four systems with different molecular weights: the FK506 binding protein (FKBP, MW = 12 kDa), Bcl-xL (MW = 19 kDa), maltose binding protein (MBP, MW = 42 kDa), and dihydroneopterin aldolase (DHNA, MW = 110 kDa).

Results and Discussion

Selection and Preparation of Precursors. One approach to preparing bacterially expressed proteins with selectively labeled methyl groups is to supplement the growth media with labeled amino acids (e.g., uniformly ^{13}C-labeled alanine, valine, etc.) that are available from commercial sources. However, this approach is expensive due to the cost of the labeled amino acids. Recently, an elegant protocol was developed for the incorporation of protonated methyl groups for valine, leucine, and isoleucine (δ1 only) in perdeuterated, uniformly ^{13}C- and ^{15}N-

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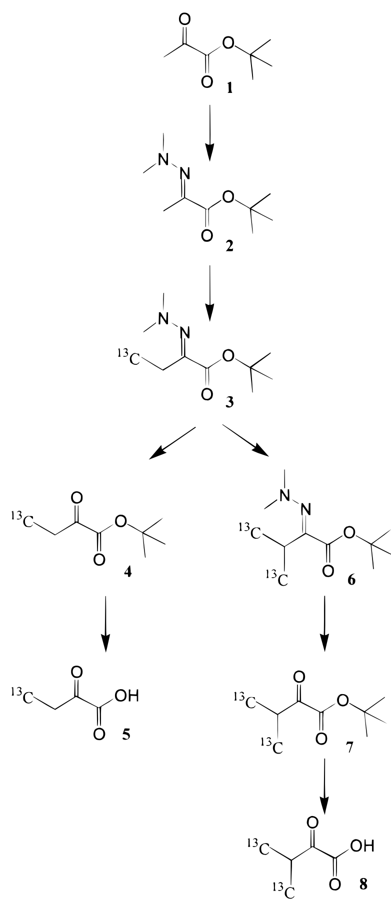
Scheme 1



labeled proteins.[5-7] This was accomplished by utilizing the isotopically labeled precursors α-ketobutyrate and α-ketoisovalerate. When added to the growth media, these compounds are biosynthetically transformed, specifically and efficiently, into isoleucine and valine/leucine, respectively. Although uniformly ^{13}C-labeled α-ketobutyrate and α-ketoisovalerate are commercially available from Cambridge Isotopes, they are expensive ($815/100 mg of U-^{13}C-α-ketobutyrate and $990/250 mg of U-^{13}C-α-ketoisovalerate) which precludes the use of these precursors for the large-scale production of isotopically labeled proteins for NMR-based screening. Furthermore, the protonated methyl groups of proteins prepared with uniformly ^{13}C-labeled precursors will exhibit ^{13}C−^{13}C coupling, which is undesirable for NMR-based screening.

To achieve cost-effective ^{13}C labeling of the methyl groups of valine, leucine, and isoleucine and remove the ^{13}C−^{13}C coupling, an efficient synthetic strategy for the production of [3-^{13}C]-α-ketobutyrate (**5**) and [3,3′-^{13}C]-α-ketoisovalerate (**8**) was developed (Scheme 1). These compounds were obtained by single or double alkylation of the *N*,*N*-dimethylhydrazone (**2**) of pyruvate *tert*-butyl ester (**1**) using ^{13}C-methyl iodide. The alkylation was followed by hydrolysis to the α-ketoester and removal of the *tert*-butyl group. The precursors **5** and **8** were prepared in approximately 47% yield from **2** in 3 and 4 steps, respectively. Using the conditions in the Experimental Section, 0.22 g (2.1 mmol) of **5** or 0.15 g (1.3 mmol) of **8** was obtained from 1 g (7 mmol) of ^{13}C-methyl iodide. This efficient route for the production of these precursors and the low cost of ^{13}C-methyl iodide makes it practical to produce large quantities of selectively ^{13}C(methyl)-labeled proteins for NMR-based screening.

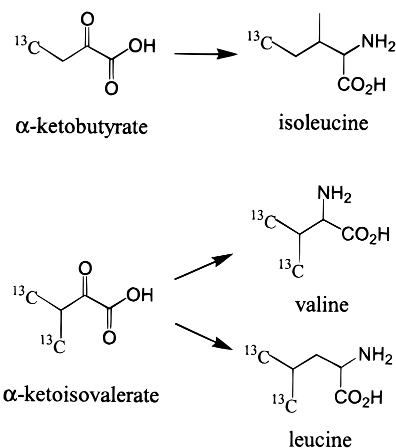


Figure 1. Biosynthetic conversion of selectively labeled α-ketobutyrate to isoleucine and α-ketoisovalerate to valine and leucine. The ^{13}C-labeled methyl group incorporated into each amino acid using the selectively labeled precursors is indicated.

Incorporation Levels. To determine the sensitivity increases that can be obtained using ^{13}C(methyl) as compared with ^{15}N labeling, samples of FKBP, Bcl-xL, and maltose binding protein (MBP) were prepared that were ^{13}C labeled at the methyl groups of valine, leucine, and isoleucine (δ1 only) and uniformly ^{15}N labeled (^{13}C(methyl)/U-^{15}N labeled). Labeling of the methyl groups was achieved by the addition of [3-^{13}C]-α-ketobutyrate (**5**) and [3,3′-^{13}C]-α-ketoisovalerate (**8**) to the growth media 30 min prior to induction with IPTG. Previous reports utilizing uniformly ^{13}C-labeled precursors in a perdeuterated environment indicated that precursor levels of approximately 50 mg/L of α-ketobutyrate and 100 mg/L of α-ketoisovalerate were sufficient for >90% incorporation at the methyl groups of valine, leucine, and isoleucine (δ1 only).[7] However, using these precursor concentrations, we observed incorporation levels of only 50−80% when using H_2O vs D_2O (as determined from one-dimensional ^{1}H NMR spectra acquired with and without ^{13}C decoupling). When the precursor concentrations were increased to 100 mg/L of **5** and 200 mg/L of **8**, incorporation levels of >90% were achieved for the methyl groups of FKBP and Bcl-xL in H_2O media. Even higher precursor concentrations (150 mg/L of **5** and 300 mg/L of **8**) were required for >90% incorporation in MBP.

NMR-Based Screening of Smaller Proteins (<30 kDa). To determine the relative sensitivity gains of ^{13}C- vs ^{15}N-based screening of smaller proteins, ^{1}H/^{13}C-HSQC and ^{1}H/^{15}N-HSQC spectra were recorded on a 500 MHz spectrometer, and the signal-to-noise (S/N) ratios for each sample were compared (Table 1). For this analysis, all S/N ratios were corrected for sample concentration and acquisition time and normalized to the S/N ratio observed for the ^{13}C/^{1}H-HSQC spectrum of FKBP. As shown in Table 1, the sensitivity of the ^{1}H/^{13}C-HSQC spectra for FKBP and Bcl-xL are nearly 3-fold higher than that of the corresponding ^{1}H/^{15}N-HSQC spectra. Thus, for proteins with molecular weights less than about 30 kDa, NMR-based screening using proteins with ^{13}C-labeled methyl groups substantially increases the sensitivity. This allows for ^{1}H/^{13}C-HSQC spectra to be acquired on a 50 μM sample of protein in 10 min, as demonstrated for Bcl-xL (Figure 2A). ^{1}H/^{15}N-HSQC spectra

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Table 1. Sensitivity Comparisons for ^{13}C(Methyl)- and ^{15}N-Labeled Proteins

protein	MW[a] (kDa)	form	^{1}H frequency (MHz)	T_1 (s)	experiment	S/N	^{13}C/^{15}N ratio
FKBP	12	^{13}C(methyl)/U-^{15}N	500	^{1}H{^{13}C}: 0.43	^{13}C-HSQC	1.00	
			500	^{1}H{^{15}N}: 0.72	^{15}N-HSQC	0.33	3.0
Bcl-xL	19	^{13}C(methyl)/U-^{15}N	500	^{1}H{^{13}C}: 0.50	^{13}C-HSQC	1.11	
			500	^{1}H{^{15}N}: 0.58	^{15}N-HSQC	0.41	2.7
MBP	42	^{13}C(methyl)/U-^{15}N	500	^{1}H{^{13}C}: 0.40	^{13}C-HSQC	0.05	
			500	^{1}H{^{15}N}: 0.39	^{15}N-HSQC	0.01	4.4
MBP	42	^{13}C(methyl)/U-^{15}N,^{2}H	500	^{1}H{^{13}C}: 0.65	^{13}C-HSQC	0.29	
			500	^{1}H{^{15}N}: 0.87	^{15}N-HSQC[b]	0.07	4.1
			800	^{1}H{^{13}C}: 0.72	^{13}C-HSQC	0.60	
			800	^{1}H{^{15}N}: 1.15	^{15}N-HSQC[b]	0.18	3.3
			800		^{15}N-TROSY	0.17	3.6
MBP	42	U-^{15}N,^{2}H	800	^{1}H{^{15}N}: 1.30	^{15}N-HSQC[b]	0.19	3.1
			800		^{15}N-TROSY	0.18	3.3
DHNA	110[c]	^{13}C(methyl)/U-^{15}N,^{2}H	500(cryo)[d]	^{1}H{^{13}C}: 0.47	^{13}C-HSQC	0.10[e]	
			500(cryo)[d]	^{1}H{^{15}N}: 0.69	^{15}N-HSQC[b]	0.014[e]	6.9
			800	^{1}H{^{13}C}: 0.58	^{13}C-HSQC	0.08[e]	
			800	^{1}H{^{15}N}: 0.72	^{15}N-HSQC[b]	0.006[e]	14.4
			800		^{15}N-TROSY	0.008[e]	9.8

[a] Molecular weight for the unlabeled protein. [b] Sensitivity-enhanced and nonsensitivity-enhanced ^{1}H/^{15}N-HSQC spectra yielded comparable results. [c] Molecular weight for the DHNA octamer. [d] Spectra and resulting signal-to-noise ratios were obtained using cryogenic probe technology.[4] [e] For comparisons between DHNA and the other proteins, no correction was made for the fact that the experiments on DHNA were performed at 277 and 285 K, as opposed to at 310 K for FKBP, Bcl-xL, and MBP. This has no effect on the observed ^{13}C/^{15}N ratios for DHNA.

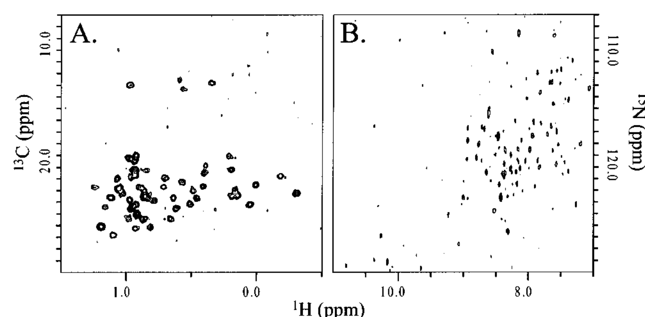

Figure 2. (A) ^{1}H/^{13}C-HSQC and (B) ^{1}H/^{15}N-HSQC spectra acquired at 500 MHz in 10 min on a 50 μM sample of ^{13}C(methyl)/U-^{15}N-labeled Bcl-xL. The ^{1}H/^{13}C-HSQC spectrum shown in (A) is suitable for NMR-based screening, while the corresponding ^{1}H/^{15}N-HSQC spectrum shown in (B) is not.

suitable for screening cannot be obtained in a time this short (Figure 2B) unless a cryoprobe is used.[4] As previously demonstrated, the ability to acquire HSQC spectra in 10 min on 50 μM protein samples can allow NMR-based screening rates of up to 10 000 compounds per day.[4] The use of ^{13}C-labeled methyl groups enables these screening rates to be obtained without the use of a cryoprobe, and even further improvements are expected with the combination of ^{13}C(methyl) labeling and this new probe technology.

NMR-Based Screening of Larger Proteins (>40 kDa). MBP (MW = 42 kDa) and DHNA (MW = 100 kDa) were used as test cases to determine whether the ^{13}C(methyl) labeling strategy would enable the NMR-based screening of larger proteins. Sensitivity gains of more than 4-fold were observed for ^{1}H/^{13}C- vs ^{1}H/^{15}N-HSQC spectra using ^{13}C(methyl)/U-^{15}N-labeled MBP (Table 1). However, the overall sensitivity was nearly 20-fold less than that obtained with the smaller proteins FKBP and Bcl-xL (Table 1). This sensitivity is insufficient to allow the rapid acquisition of NMR spectra for large proteins. Since significant increases in sensitivity have been observed through the use of deuterium labeling,[8] methyl-protonated, perdeuterated, uniformly ^{15}N-labeled samples of MBP and DHNA were prepared by growing in 100% D_2O media with U-^{2}H-glucose and the precursors **5** and **8** as the sole carbon sources (hereafter denoted as ^{13}C(methyl)/U-^{15}N,^{2}H-labeled). In these cases, precursor concentrations of 50 mg/L of **5** and 100 mg/L of **8** were sufficient for >90% incorporation of the ^{13}C-labeled methyl groups. A comparison of ^{1}H/^{13}C-HSQC spectra acquired on MBP at 500 MHz on the protonated and deuterated samples indicates a 6-fold increase in sensitivity as a result of the deuteration (Table 1). Furthermore, the sensitivity of the ^{1}H/^{13}C-HSQC experiment on the ^{13}C(methyl)/U-^{15}N,^{2}H-labeled MBP sample was more than a factor of 4 greater than that of the ^{1}H/^{15}N-HSQC experiment. For the ^{13}C(methyl)/U-^{15}N,^{2}H-labeled DHNA sample, the ^{1}H/^{13}C-HSQC spectrum was nearly a factor of 7 more sensitive than the ^{1}H/^{15}N-HSQC spectrum (Table 1). In fact, ^{1}H/^{13}C-HSQC spectra which are suitable for NMR-based screening could be obtained in less than 30 min on this same sample (Figure 3).

Comparison to TROSY. It has recently been demonstrated that the use of transverse relaxation-optimized spectroscopy (TROSY) can yield substantial sensitivity gains over that of the ^{15}N/^{1}H-HSQC experiment for larger, deuterated systems at higher fields.[9] To investigate the sensitivity comparisons between the ^{1}H/^{13}C-HSQC and ^{1}H/^{15}N-TROSY-type experiments, spectra were acquired on the ^{13}C(methyl)/U-^{15}N,^{2}H-labeled MBP and DHNA samples at 800 MHz. For MBP, the ^{1}H/^{15}N-TROSY experiment was more than 3-fold less sensitive than the ^{1}H/^{13}C-HSQC experiment (Table 1). This is dramatically illustrated in Figure 4, where a ^{1}H/^{13}C-HSQC spectrum acquired in 10 min on a 50 μM sample of ^{13}C(methyl)/U-^{15}N,^{2}H-labeled MBP is clearly more sensitive than a ^{1}H/^{15}N-TROSY spectrum acquired on the same sample. Comparable signal-to-noise estimates were obtained for both the ^{13}C(methyl)/U-^{15}N,^{2}H-labeled and U-^{15}N,^{2}H-labeled MBP (Table 1), suggesting that protonated methyls in a deuterated environment do not significantly affect the sensitivity of the ^{1}H/^{15}N-TROSY or ^{1}H/^{15}N-HSQC experiments. For the ^{13}C(methyl)/U-^{15}N,^{2}H-labeled DHNA sample, the ^{1}H/^{13}C-HSQC experiment was nearly 10-fold more sensitive than the ^{1}H/^{15}N-TROSY experiment and a factor of 15 more sensitive than the ^{1}H/^{15}N-HSQC experiment

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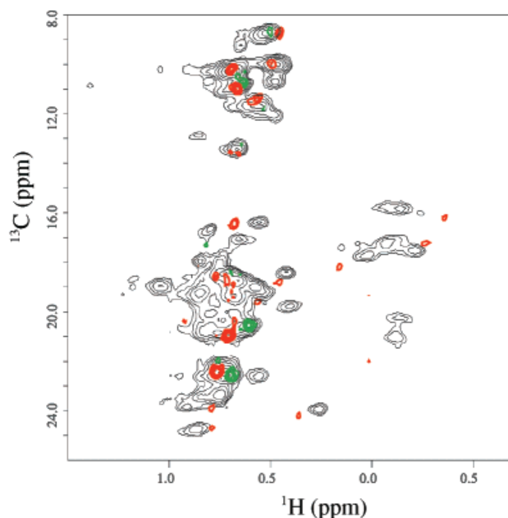

Figure 3. $^{1}H/^{13}C$-HSQC spectra acquired on a 500 MHz spectrometer equipped with a Cryoprobe on a 0.3 mM sample of ^{13}C(methyl)/U-^{15}N,^{2}H-labeled DHNA (black contours). Also shown are the results of differencing spectra acquired in the presence and absence of D(+)-neopterin. Red contours indicate signal intensity which was reduced with the ligand, while green contours indicate increased signal intensity.

(Table 1). Furthermore, to obtain the full benefit of the TROSY experiment, very high magnetic fields are required (typically 800 MHz or higher). In contrast, our approach using ^{13}C-(methyl)-based screening can be effectively performed on 500 MHz NMR spectrometers that are less expensive and easily outfitted with automatic sample changers. Thus, ^{13}C(methyl) labeling strategies which include deuterium labeling are superior to ^{15}N-based approaches for the screening of large protein targets.

Detection of Ligand Binding. The ability to detect ligand binding using ^{13}C(methyl)-labeled samples is shown in Figure 5 for FKBP binding to 2-phenylimidazole. Using $^{1}H/^{15}N$-HSQC spectra, significant chemical shift changes are observed for the backbone amide groups of F36, S38, F48, L50, G51, V55, I56, W59, E61, A64, I90, and F99 (Figure 5A). All of the valine, leucine, and isoleucine residues that exhibit significant chemical shift changes in $^{1}H/^{15}N$-HSQC spectra (L50, V55, I56, and I90) also exhibit chemical shift changes in $^{1}H/^{13}C$-HSQC spectra (Figure 5B). In addition, chemical shift changes are observed for the methyl groups of V24, V63, and I76. All of these residues are located in or near the ascomycin binding site of FKBP, and the binding site could be identified from an analysis of the changes observed in either the $^{1}H/^{15}N$- or $^{1}H/^{13}C$-HSQC spectra. $^{1}H/^{13}C$-HSQC spectra could also be used to detect NMR binding between ^{13}C(methyl)/U-^{15}N,^{2}H-labeled DHNA and D(+)-neopterin. In this case, subtracting the $^{1}H/^{13}C$-HSQC spectra acquired in the absence and presence of 1.0 mM neopterin clearly revealed chemical shift changes even in the highly overlapped portions of the spectrum (Figure 3).

With the selective labeling strategy employed here, proteins whose active sites do not contain valine, leucine, or isoleucine residues may not exhibit chemical shift changes upon ligand binding. However, these three residues are commonly found to comprise the hydrophobic sites of proteins. In fact, in an analysis of 191 nondegenerate crystal structures of proteins bound to organic molecules, 92% of the ligands were found to have a heavy atom within 6 Å of at least one methyl carbon of valine, leucine, or isoleucine (δ1 only). In contrast, only 82% of the ligands were found within 6 Å of at least one backbone nitrogen. In addition, even slight conformational or electronic changes in the protein which occur upon complex formation can give rise to observable chemical shift changes even for atoms which do not interact directly with the ligand. For example, in the case of phenylimidazole binding to FKBP, the methyl groups of L50 are significantly perturbed upon complex formation, even though the methyl groups of this residue are more than 9 Å away from the ligand. The perturbations in the chemical shifts of L50 are likely due in part to conformational changes in W59, which forms intimate contacts with the ligand and is proximal to L50. Thus, ^{13}C(methyl)-based screening should be a reliable method for the detection of ligand binding to a wide variety of proteins.

Conclusions

The sensitivity gains realized with the ^{13}C(methyl)-labeling strategy described in this manuscript can dramatically impact the efficiency of NMR-based screening. For small to moderately sized proteins (MW $\leq$ 30 kDa), ^{13}C(methyl)-based screening should yield increases in signal-to-noise of nearly a factor of 3 as compared with ^{15}N-based screening. This will allow the rapid acquisition of $^{1}H/^{13}C$-HSQC spectra even on very dilute protein samples. In fact, when used in combination with the recent advances in cryogenic probe technology, NMR-based screening should be possible with protein concentrations as low as 15 μM. Furthermore, this labeling strategy is cost-effective. It is estimated that the labeled media cost per gram of cell paste is \$5.85 for ^{13}C(methyl) labeling, as compared with \$5.40 for uniform ^{15}N labeling, \$64.00 for uniform ^{13}C labeling, and \$129.00 for ^{13}C(methyl) labeling with commercially available U-^{13}C-α-ketobutyrate and U-^{13}C-α-ketoisovalerate.[10]

For larger proteins (MW $\geq$ 40 kDa), ^{13}C(methyl)-based screening is much more sensitive than ^{15}N-based methods, even when compared with the recently introduced $^{1}H/^{15}N$-TROSY experiments. Although the protein must be deuterated to reduce the line broadening caused by dipolar relaxation, the large gains in sensitivity make the screening of large proteins practical. Indeed, we have shown that it will be possible to screen proteins as large as 100 kDa using our approach. Thus, ^{13}C(methyl)-based screening promises to be a useful tool in drug research.

Experimental Section

Pyruvate-*tert*-butyl ester, *N*,*N*-dimethylhydrazone (2). The pyruvate *tert*-butyl ester **1** (77.3 g, 536 mmol) was dissolved in 100 mL of diethyl ether, dimethyl hydrazine (31.6 g, 525 mmol, 40 mL, 0.98 equiv) was added dropwise over 20 min, and the reaction was stirred overnight at ambient temperature. TLC (25% EtOAc/hexanes) showed no starting material, and the diethyl ether was evaporated and the product distilled under reduced pressure (16 mmHg, 94−99 degrees C) to give 92.1 g (92%) of **2** as a yellow oil as a mixture of syn/anti hydrazones. ^{1}H NMR (300 MHz, $CDCl_3$): mixture of syn/anti *N*,*N*-dimethylhydrazones δ 2.80 and 2.51 (together 6H), 2.07 and 2.05 (together 3H), 1.53 and 1.52 (51 (together 9H). LRMS $C_9H_{18}N_2O_2$ (APCI + Q1MS): 187.

3-^{13}C-Methyl-2-oxo-*tert*-butylpropionate, *N*,*N*-dimethylhydrazone (3). To a three-neck flask equipped with an addition funnel was added 100 mL of anhydrous THF by cannula. Diisopropylamine (7.51 g, 74.2 mmol, 10.4 mL, 1.35 equiv, freshly distilled from CaH_2) was added by syringe. The solution was cooled to −78 °C, and to the solution was added a dropwise addition of *n*-BuLi (28.3 mL of a 2.5 M soln in

(10) Assuming a fermentor growth with induction at OD_{600} = 2.0, the required amount of ^{13}C-glucose, $^{15}NH_4Cl$, or α-ketoisovalerate/α-ketobutyrate per liter of media is 4, 1.5, or 0.2/0.1 g, respectively. Costs are estimated assuming the production of 10 g of cell paste per liter. Isotope costs of \$142/g for ^{13}C-glucose (CIL), \$30/g for $^{15}NH_4Cl$ (CIL), \$815/100 mg for U-^{13}C-α-ketobutyrate (CIL), \$900/250 mg for U-^{13}C-α-ketoisovalerate (CIL), and \$293 for 1 g of **5** and 0.5 g of **8** (based on \$33.50/g for $^{13}CH_3I$ (Aldrich) and the reported synthetic yields for the reaction shown in Scheme 1) were used.

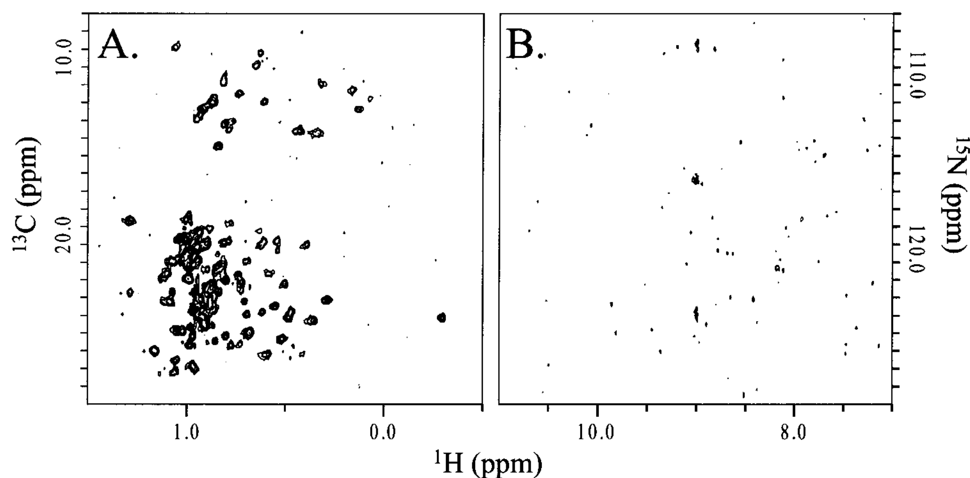

Figure 4. (A) ^{1}H/^{13}C-HSQC and (B) ^{1}H/^{15}N-TROSY spectra acquired at 800 MHz in 10 min on a 50 μM sample of ^{13}C(methyl)/U-^{15}N,^{2}H-labeled MBP. The ^{1}H/^{13}C-HSQC spectrum shown in (A) is suitable for NMR-based screening, while the corresponding ^{1}H/^{15}N-TROSY spectrum shown in (B) is not.

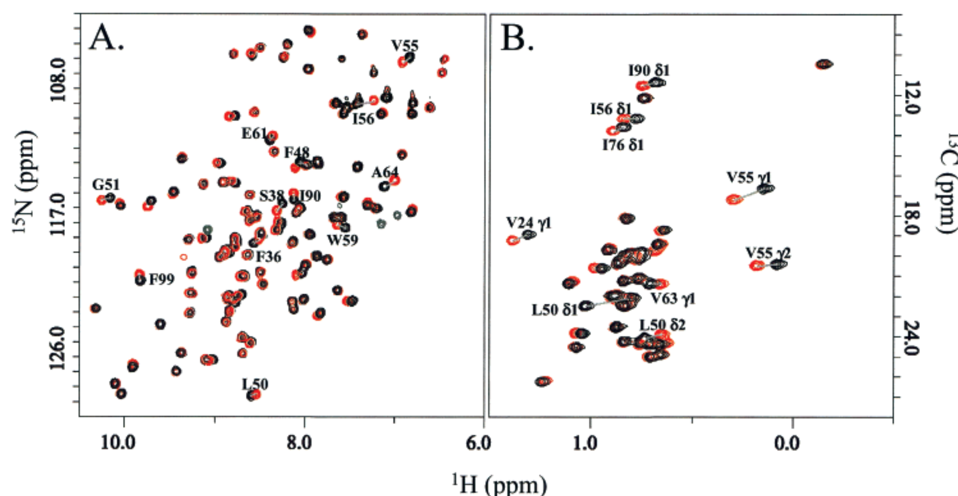

Figure 5. (A) ^{1}H/^{15}N-HSQC and (B) ^{1}H/^{13}C-HSQC spectra acquired at 500 MHz on a 0.35 mM sample of FKBP in the absence (black contours) and presence (red contours) of 0.4 mM phenylimidazole.

hexanes, 70.7 mmol, 1.3 equiv) by addition funnel over 10 min. The reaction was warmed to 0 °C for 5 min and then cooled back to −78 °C. The addition funnel was rinsed with 5 mL of THF, and then a solution of hydrazone **2** (10.1 g, 54.4 mmol, 1.0 equiv) in 40 mL of THF was added dropwise by addition funnel over 20 min. The enolate (dark in color) was allowed to generate over 1 h at −78 °C and was then transferred by cannula to another 500-mL flask also at −78 °C containing $^{13}CH_3I$ (11.7 g, 81.6 mmol, 5.1 mL, 1.5 equiv) in 50 mL of THF over 10 min. The reaction was stirred at −78 °C for 2 h and was quenched with 10 mL of H_2O, warmed to room temperature, evaporated, and taken up in 200 mL of EtOAc. This EtOAc solution was washed with H_2O (50 mL × 2), dried over Na_2SO_4, filtered, and evaporated to a brown oil (crude NMR shows clean desired product) which was purified by flash chromatography (50% EtOAc/hexanes) to give 8.21 g (75%) of **3** as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.78 (s, 6H), 2.61−2.51 (m, methylenes split by ^{13}C−H coupling, 2H), 1.53 (s, 9H), 1.33 and 0.90 (two t, $J = 7.5$ and 7.7 Hz, respectively, together 3H; ^{13}C−H coupling is 127.6 Hz). LRMS $^{13}C_1C_9H_{20}N_2O_2$ (APCI, MH$^+$) 202.

3-^{13}C-Methyl-2-oxo-*tert*-butylpropionate (4). Crude ^{13}C-labeled hydrazone **3** (9.19 g, 45.7 mmol) was dissolved in 165 mL of THF, and 55 mL of 1 N HCl (1.2 equiv) was added and the reaction was stirred at room temperature. TLC (25% EtOAc/hexanes) at 45 min showed complete reaction. The reaction was diluted with 400 mL of Et_2O and the layers separated. The aqueous layer was extracted with Et_2O (50 mL × 3). The Et_2O layers were combined, washed with saturated brine, dried over Na_2SO_4, filtered, evaporated to an oil, and purified by flash chromatography (30% Et_2O/pentanes) to give 5.54 g of **4** as a light yellow oil (76% over 2 steps from **2**). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.85−2.76 (m, methylenes split by ^{13}C−H coupling, 2H), 1.55 (s, 9H), 1.33 and 0.90 (two t, $J = 7.0$ and 7.0 Hz, respectively, together 3H; ^{13}C−H coupling is 128.7 Hz). LRMS $^{13}C_1C_7H_{14}O_3$ (APCI, MH$^+$NH$_4$): 177.

3-^{13}C-Methyl-2-oxo-propionic acid (5). The *tert*-butyl ester **4** (5.54 g, 34.8 mmol) was dissolved in 200 mL of a 1:1 mixture of CH_2Cl_2/Et_2O and cooled to 0 °C, and anhydrous HCl gas was bubbled in for 2 min at a moderate rate. The reaction was sealed with a yellow cap to keep the HCl gas contained under a slight amount of pressure as it came to room temperature and the reaction was stirred overnight. TLC showed reaction to be incomplete. The reaction was recooled to 0 °C, and HCl gas was again bubbled in, this time for 5 min at a moderate rate, and the reaction was again allowed to warm to room temperature. The reaction was complete 6 h after this second HCl treatment and was evaporated to an oil and purified by flash chromatography (100% Et_2O, short silica gel column) to give 2.21 g (62%) of **5** as a clear oil that solidifies in the freezer. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.04−2.95 (m, methylenes split by ^{13}C−H coupling, 2H), 1.39 and 0.96 (two t, $J = 7.0$ and 7.0 Hz, respectively, together 3H, ^{13}C−H coupling is 128.7 Hz). LRMS $^{13}C_1C_3H_6O_3$ (APCI, MH$^-$) 102.

3,3-Di-^{13}C-methyl-2-oxo-*tert*-butylpropionate, *N*,*N*-dimethylhydrazone (6). To a three-neck flask equipped with an addition funnel was added 100 mL of anhydrous THF by cannula. Diisopropylamine (5.64 g, 55.8 mmol, 7.8 mL, 1.35 equiv, freshly distilled from CaH_2) was added by syringe. The solution was cooled to −78 °C and to the

solution was added, dropwise, *n*-BuLi (21.2 mL of a 2.5 M soln in hexanes, 53.1 mmol, 1.3 eq.) by addition funnel over 10 min. The reaction was warmed to 0 °C for 5 min and then cooled back to −78 °C. The addition funnel was rinsed with 5 mL of THF, and then a solution of hydrazone **3** (8.21 g, 40.8 mmol, 1.0 equiv) in 40 mL of THF was added dropwise by addition funnel over 20 min. The enolate was allowed to generate over 1 h at −78 °C and was then transferred by cannula to another 500-mL flask also at −78 °C containing $^{13}CH_3I$ (8.74 g, 61.2 mmol, 3.83 mL 1.5 equiv) in 50 mL of THF over a period of 10 min. The reaction was stirred at −78 °C for 2 h and was quenched with 10 mL of H_2O, warmed to room temperature, evaporated, and taken up in 200 mL of EtOAc. This EtOAc solution was washed with H_2O (50 mL × 2), dried over Na_2SO_4, filtered, and evaporated to give **6** as a brown oil (crude NMR shows clean desired product) which was taken on without further purification. ^1H NMR (300 MHz, $CDCl_3$): δ 2.47 and 2.35 (two *N*,*N*-dimethyl singlets from E/Z hydrazones, total 6H), 1.54 and 1.52 (two *tert*-butyl singlets from E/Z hydrazones, total 9H), 1.43 and 1.34 (two m (dd that are complicated by E/Z hydazones), together 6H). LRMS: $^{13}C_2C_9H_{22}N_2O_2$ (APCI, MH^+) 217.

3,3-Di^{13}C-methyl-2-oxo-*tert*-butylpropionate (7). The dialkylated hydrazone **6** (8.89 g, 41 mmol, assume 100% from **3**) was dissolved in 150 mL of THF, and 49 mL of 1 N HCl (1.2 equiv) was added and the reaction was stirred at room temperature. TLC (25% EtOAc/hexanes) at 1 h showed complete reaction. The reaction was poured into a separatory funnel and diluted with 500 mL of Et_2O and the layers separated. The aqueous layer was extracted with Et_2O (50 mL × 3). The Et_2O layers were combined, washed with 100 mL of saturated brine, dried over Na_2SO_4, filtered, evaporated to an oil, and purified by flash chromatography (30% Et_2O/pentanes) to give 5.44 g of **7** as a light yellow oil (76% over 2 steps from **3**). ^1H NMR (300 MHz, $CDCl_3$): δ 3.23−3.13 (m, methine split by $^{13}C-H$ coupling, 1H), 1.55 (s, 9H), 1.36 and 0.93 (two dd, $J = 6.8$, 4.1 Hz for each dd, together 6H, $^{13}C-H$ coupling is 128.2 Hz). LRMS $^{13}C_2C_7H_{16}O_3$ (APCI, MH^+-NH_4): 192.

3,3-Di ^{13}C-methyl-2-oxo-propionic acid (8). The *tert*-butyl ester **7** (5.44 g, 31.2 mmol) was dissolved in 200 mL of a 1:1 mixture of CH_2Cl_2/Et_2O and cooled to 0 °C, and anhydrous HCl gas was bubbled in for 2 min at a moderate rate. The reaction was sealed with a yellow cap, to keep the HCl gas contained, and was stirred at room temperature. TLC (50% EtOAc/hexanes) at 1 h showed the reaction to be incomplete. The reaction was recooled to 0 °C, and HCl gas was again bubbled in, this time for 5 min at a moderate rate, and the reaction was again allowed to warm to room temperature. The reaction was monitored every hour by TLC and was retreated with HCl each time until it was complete after 4 h. The reaction was evaporated to an oil and purified by flash chromatography (50% Et_2O/pentanes, short silica gel column) to give 3.10 g (84%) of **8** as a clear oil that crystallized upon standing. ^1H NMR (300 MHz, $CDCl_3$): δ 3.58−3.40 (m, methine split by $^{13}C-H$ coupling, 1H), 1.43 and 1.01 (two dd, $J = 7.0$, 5.2 Hz for each dd, together 6H, $^{13}C-H$ coupling is 129.1 Hz). LRMS $^{13}C_2C_3H_8O_3$ (APCI, MH−): 117.

Protein Preparation. FKBP and Bcl-xL were overexpressed in *E. coli* as previously described.[11,12] MBP was prepared using the pMAL-c2 vector (New England Biolabs). The double stranded DNA encoding DHNA was isolated by PCR amplification from *Staphylococcus aureus* genomic DNA using oligodeoxynucleotide primers based on the DHNA gene sequence (preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org). The gene was cloned into the NdeI-XhoI restriction sites of the expression vector pET-30b (Novagen). A TAA-stop codon was inserted between the last codon of the protein and any C-terminal tail so that only the native protein sequence was translated. All vector sequences were confirmed by bidirectional dye-terminator DNA sequencing. In all growths, $^{15}NH_4Cl$ (Cambridge Isotopes) was included as the sole nitrogen source. The amino acid precursors [3-^{13}C]-α-ketobutyrate (**5**) and [3,3′-^{13}C]-α-ketoisovalerate (**8**) were added 30 min prior to induction with 1 mM IPTG. For FKBP and Bcl-xL growths in H_2O media, precursor concentrations of 100 mg/L of **5** and 200 mg/L of **8** were required for >90% incorporation, while 150 mg/L of **5** and 300 mg/L of **8** were required for >90% incorporation in MBP. Incorporation levels for protonated samples were monitored by acquiring one-dimensional ^1H NMR spectra with and without ^{13}C decoupling and monitoring resolved methyl signals. For all growths in D_2O media, the precursor concentrations of 50 mg/L of **5** and 100 mg/L of **8** were sufficient for >90% incorporation (as determined my mass spectral analysis of the hydrolyzed protein). Deuterium incorporation levels under these conditions were greater than 98%. The constructs for both FKBP and Bcl-xL contain C-terminal 6-His tags, and the proteins were purified in a single step by affinity chromatography using Ni^{2+} resin (Invitrogen) and eluting with 500 mM imidazole. Samples of MBP were purified in a single step by affinity chromatography using amylose resin (New England Biolabs) and eluting with 10 mM maltose. DHNA was purified from BL21(DE3) cells using the reported procedure.[13] The final buffer conditions were the following. FKBP: 50 mM Na_2PO_4, 100 mM NaCl, pH 7.0. Bcl-xL: 20 mM Na_2PO_4, pH 7.0. MBP: 20 mM Na_2PO_4, 1 mM maltose, pH 7.0. DHNA: 20 mM MES, pH 7.0.

NMR Experiments. For FKBP, Bcl-xL, and MBP, $^1H/^{13}C$-HSQC, sensitivity-enhanced $^1H/^{15}N$-HSQC with a water flip-back sequence,[14] and $^1H/^{15}N$-TROSY[9,15] spectra were recorded at 310 K on Bruker DRX500 and DRX800 spectrometers. In addition, nonsensitivity-enhanced $^1H/^{15}N$-HSQC spectra were recorded for MBP. FOR DHNA, $^1H/^{13}C$-HSQC, sensitivity- and nonsensitivity-enhanced $^1H/^{15}N$-HSQC, and $^1H/^{15}N$-TROSY spectra were recorded at 277 K (800 MHz) and 285 K (500 MHz equipped with a Cryoprobe). All spectra were recorded with 64 complex points and processed offline using in-house written software. A total recycle delay of 1.2 s was used for all two-dimensional HSQC experiments.[16] Data were linear predicted to 94 complex points before Fourier transformation. For the signal-to-noise comparisons given in Table 1 for FKBP and Bcl-xL, spectra were recorded on a 0.6−1.0 mM samples with 4 scans/increment (10 min acquisition time). For the signal-to-noise comparisons for ^{13}C(methyl)/U-^{15}N,^{2}H-labeled MBP at 500 and 800 MHz, spectra were recorded on 0.32 mM samples using 24 scans/increment (1 h acquisition time). For ^{13}C(methyl)/U-^{15}N-labeled MBP, total acquisition times on a 0.32 mM sample were 15 h (360 scans/increment) and 4 h (96 scans/increment) for spectra acquired at 500 and 800 MHz, respectively. For the signal-to-noise comparisons for ^{13}C(methyl)/U-^{15}N,^{2}H-labeled DHNA, spectra were recorded on a sample which was 0.3 mM in monomer (37.5 μM octamer). Data were collected on a 500 MHz spectrometer equipped with a Cryoprobe (Bruker) using 12 scans/increment (30 min acquisition time) for the $^1H/^{13}C$-HSQC experiment and 384 scans/increment (16 h acquisition time) for the $^1H/^{15}N$-HSQC experiment. Data were also collected on an 800 MHz spectrometer using 96 scans/increment (4-h acquisition time) for the $^1H/^{13}C$-HSQC experiment, 512 scans/increment (21.2 h acquisition time) for the $^1H/^{15}N$-TROSY experiment, and 256 scans/increment (10.6 h acquisition time) for the $^1H/^{15}N$-HSQC experiment. Proton T_1 estimates were obtained using one-dimensional versions of the $^1H/^{13}C$-HSQC and $^1H/^{15}N$-HSQC experiments with an inversion−recovery sequence ($180 - \tau -$) inserted after a 10-s recovery delay, where τ was varied until a null in the observed proton magnetization was obtained ($T_1 = \tau_{null}/\ln 2$).

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(16) For the proteins used in this analysis, variations in recycle time will have little effect on the signal-to-noise ratios for $^1H/^{13}C$ and $^1H/^{15}N$ experiments. This was tested empirically for Bcl-xL, in which signal-to-noise estimates for $^1H/^{13}C$ and $^1H/^{15}N$−HSQC spectra were obtained using recycle delays of 0.5−2.0 s and adjusting the number of scans to keep the total acquisition time constant. The ratios of signal-to-noise between the $^1H/^{13}C$ and $^1H/^{15}N$ spectra were virtually identical ($^{13}C/^{15}N$ ratio ∼3.0) in all cases.

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Data Analysis. The signal-to-noise ratio for each spectrum was estimated by dividing the average peak height by the observed noise in the two-dimensional spectrum. An initial average peak height was obtained by including all observable peaks in the spectrum. The final average peak height excluded all peaks whose heights were greater than four times the average value. The average numbers of peaks used to calculate peak heights were 44 and 116 for the carbon and nitrogen spectra, respectively. Spectral noise was defined as the standard deviation in the heights of a set of random positions in the spectrum (excluding areas with observable cross peaks or spectral artifacts). The average number of positions used to calculate the spectral noise was 50. Signal-to-noise estimates were normalized for sample concentration by using a linear relationship between the observed signal-to-noise and the protein concentration. The signal-to-noise estimates were also normalized for acquisition time by using a square-root dependence of the signal-to-noise on the total acquisition time (e.g., signal-to-noise $\propto \sqrt{\text{time}}$).[17]

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