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Methyl Side-Chain Dynamics in Proteins Using Selective Enrichment with a Single Isotopomer

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Abstract: ^{13}C relaxation studies on side-chain methyl groups in proteins typically involve measurements on $^{13}CHD_2$ isotopomers, where the ^{13}C relaxation mechanism is particularly straightforward in the presence of a single proton. While such isotopomers can be obtained in proteins overexpressed in bacteria by use of ^{13}C enriched and fractionally deuterated media, invariably all possible ^{2}H isotopomers are obtained. This results in a loss of both resolution and sensitivity, which becomes particularly severe for larger proteins. We describe an approach that overcomes this problem by chemical synthesis of amino acids containing a pure $^{13}CHD_2$ isotopomer. We illustrate the benefits of this approach in ^{13}C side-chain relaxation measurements on the mouse major urinary protein selectively enriched with [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^{2}H_6$] valine. Relaxation measurements in the absence and presence of pyrazine-derived ligands suggest that valine side-chain dynamics do not contribute significantly to binding entropy.

Introduction

Molecular recognition is fundamental to all biological processes. However, despite enormous advances in structural biology over recent decades, our understanding of the principles that govern specificity and affinity in biomolecular interactions is very limited. Simple thermodynamic arguments show that the free energy of binding of two molecules is governed not only by the enthalpy (loosely, structure) of the interacting species, but also by the entropy of the system, which is in turn related to dynamics. Consequently, there is currently much interest in obtaining a measure of the elusive entropic component to binding free energy using a variety of biophysical methods, including isothermal titration calorimetry and molecular dynamics simulations.[1−10]

While the entropic component to binding is multifaceted, involving degrees of freedom of both the ligand, protein, and solvent, NMR relaxation measurements can in principle provide quantitative *per residue* information on the entropic contribution from each of these.[11−20] In particular, recent studies have focused on the entropic contribution to binding of side-chain methyl groups in proteins.[18,21−27] These studies have utilized either ^{13}C or ^{2}H relaxation measurements in $^{13}CHD_2$ or $^{13}CH_2D$ isoto-

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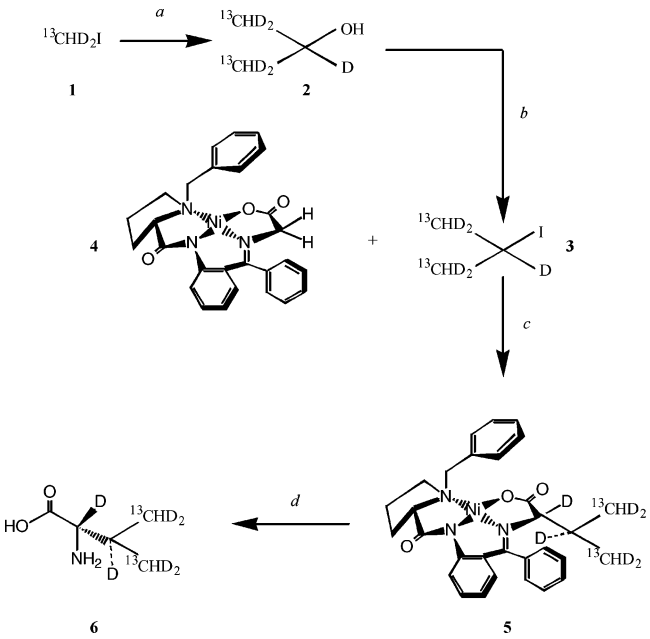
pomers, respectively. As discussed in detail by Muhandiram et al.,[21] there are compelling reasons to choose ^{2}H relaxation measurements where possible. However, ^{13}C relaxation measurements are considerably more sensitive[24] and may represent the only viable approach in larger proteins.[26] Indeed, during studies on the mouse major urinary protein (MUP) as a model system for studying the thermodynamics of ligand−protein interactions, we have experienced considerable difficulty in the measurement of accurate ^{2}H relaxation rates for valine methyl groups in methyl ^{13}C, 50% ^{2}H enriched protein due to the combined effects of resonance overlap and relatively poor sensitivity, despite the modest size of this protein (~19 kDa). The low sensitivity derives in part from the fact that only the $^{13}CH_2D$ is detected in ^{2}H relaxation measurements, whereas the sample contains all possible ^{2}H isotopomers, even with optimized labeling schemes.[28] Consequently, the effective protein concentration is reduced considerably. Unfortunately, while ^{13}C relaxation measurements on this system offered much higher sensitivity, resonance overlap was very severe due in part to the presence of contaminating resonances from $^{13}CH_3$ isotopomers in refocused INEPT experiments designed to selectively detect resonances from $^{13}CHD_2$ isotopomers.[29] The former, which resonate at a different chemical shift from the latter due to the deuterium isotope effect, are incompletely suppressed in refocused INEPT experiments as a consequence of the different relaxation rates in proteins of the 3/2 and 1/2 spin manifolds of the ^{13}C spin in CH_3 groups.

To overcome these resonance overlap difficulties, we describe a chemical synthetic approach for the preparation of valine containing a pure $^{13}CHD_2$ isotopomer, i.e., [γ^1,γ^2-$^{13}C_2$,α,β,-γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine. By inclusion of this material in an appropriate bacterial growth medium,[30] protein can be prepared which contains isotopically enriched methyl groups only from valine residues. In the case of MUP, all 12 valine methyl groups are resolved, enabling highly accurate ^{13}C relaxation times to be measured. A comparison with relaxation data obtained in the presence of the ligands 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine suggests that valine side chains do not contribute significantly to the entropy of binding.

Synthesis of [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] Valine (6). The valine isotopomer (**6**, Scheme 1) was synthesized using a (*S*)-2-[*N*-(*N*′-benzylprolyl)amino]benzophenone (BPB) nickel(II) complex as a chiral auxiliary.[31,32] The isotopically enriched isopropyl side chain was installed using the appropriate secondary iodide (**3**), which was made from [$^2H_2$, ^{13}C] methyl iodide (**1**) and methyl [^{2}H] formate via a Grignard reaction, thus giving secondary alcohol **2** which was subsequently iodinated with hydroiodic acid. The valine α-hydrogen atom was exchanged for deuterium under basic conditions prior to acidic cleavage from the auxiliary which could be recovered in high yield. Valine (**6**) was produced in an overall yield of 6% and 85% ee.

Scheme 1. Synthesis of Isotopically Enriched Valine (**6**)



[a] Reagents: (a) Mg/DCO_2Me/Et_2O. (b) HI/H_2O. (c) (i) NaO^{t}Bu/CH_3CN; (ii) Na/PhMe/MeOD. (d) 2 M HCl/MeOH.

This enantiomeric excess is adequate for biosynthetic purposes since bacteria will only metabolize the "correct" enantiomer. Full synthetic details can be found in the Supporting Information.

Expression and Purification of [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] Valine Enriched Mouse Major Urinary Protein. Full details of the expression and purification of MUP can be found in the Supporting Information. Briefly, M15 cells transformed with the vector PQE30 MUP were grown in 1 liter of medium containing initially unlabeled amino acids and nutrients. When cell density had reached an OD of 1.2, the cells were transferred to an equivalent medium but in which [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine was substituted for the unlabeled material. The concentrations of the relevant amino acids in this medium were chosen to minimize isotopic scrambling which otherwise occurs if glucose is used as the supplementary carbon source (unpublished observations). After 30 min, protein expression was induced by addition of IPTG to a final concentration of 0.1 mmol. After 6 h, the cells the culture was centrifuged at 4000 rpm for 20 min in a Sorvall RC-3B centrifuge. The cells were broken by passing them through a French press, and the broken cells were spun at 15000g for 20 min in Oakridge tubes. MUP was purified from the supernatant by use of Ni−NTA immobilized metal affinity column chromatography and further purified by Sephadex G-25 size-exclusion chromatography and Resource Q anion exchange chromatography. The protein was dialyzed exhaustively at 4 °C versus 50 mM sodium phosphate pH = 7.0. The total yield was 90 mg. Throughout this work the residue numbering of Abbate et al. was used. [33]

Expression and Purification of Methyl-^{13}C, ^{2}H Enriched Mouse Major Urinary Protein. Methyl-^{13}C, 50% ^{2}H enriched mouse major urinary protein was prepared as described in the previous section, substituting 99.9% D_2O medium containing protonated 3-^{13}C-pyruvate, as described.[34]

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^{13}C NMR Relaxation Measurements. Samples of [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine enriched MUP both alone and complexed with 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine were prepared from a single sample at pH 7.0 and a protein concentration of 1 mM. A further sample of methyl-^{13}C, ^{2}H enriched MUP was prepared at pH 7.0 and a protein concentration of 0.5 mM. Longitudinal (R_1) and transverse (R_2) ^{13}C relaxation rates were determined essentially as described by Ishima et al.[25] All spectra were recorded at a proton frequency of 600 MHz and a probe temperature of 35 °C. R_1 rates were determined with relaxation delay times of 16, 64, 128, 240, 400, 720, 1120, 1600, 2240, and 2880 ms. R_2 rates were determined with relaxation delay times of 16, 32, 48, 64, 96, 128, 160, 192, 240, and 288 ms and an effective field strength of 2 kHz. Relaxation data were fit to a single exponential decay function $I = I_o \exp(-tR)$, where I_o is the initial resonance intensity, t is the relaxation delay time, and R is the relaxation rate (R_1 or R_2). Isotropic rotational tumbling was assumed on the basis of previous backbone ^{15}N relaxation measurements for this protein.[17] Thus, relaxation data were fitted to the Lipari−Szabo model-free spectral density[35] of the form:

$$J(\omega) = S^2\tau_m/(1 + \omega^2\tau_m^2) + (1 - S^2)\tau_i/(1 + \omega^2\tau_i^2) \quad (1)$$

where $\tau_i^{-1} = \tau_M^{-1} + \tau_e^{-1}$, τ_M is the overall molecular tumbling correlation time, and τ_e is the effective internal correlation time. This equation is valid for fast internal motions, $\tau_e \ll \tau_M$, under which conditions the order parameter for methyl CH dipolar relaxation is given by $S^2 = S^2_{axis}[P_2(\cos\theta_H)]^2$. S^2_{axis} is the order parameter of the methyl rotation axis and θ_H is the angle made by this axis and the CH bond vector. The MODELFREE program was used for all these calculations,[36] kindly supplied by Prof. Art Palmer (Columbia University) and modified in-house to include relaxation of ^{13}C by the attached deuterons in $^{13}CHD_2$ isotopomers. A global rotational correlation time of 8.57 ns was used for these calculations, according to the previously reported value.[17] Errors in S^2 values were estimated by Monte Carlo simulations as implemented in the MODELFREE package. In all cases, good fits were obtained to the above spectral density function without a contribution from exchange-broadening R_{ex}. Although valine residues are perdeuterated at nonmethyl positions, the protein is otherwise protonated, and dipolar relaxation due to these "external" protons is not negligible for ^{13}C relaxation. Thus, by analogy with the work of Ishima et al.,[26] the contribution of these external protons to ^{13}C R_2 values was estimated at 25% from the X-ray coordinates of MUP (R. Bingham and S. E. V. Phillips, unpublished data). Consequently, measured ^{13}C R_2 rates were multiplied by 0.75 to account for these external dipolar relaxation processes.

Results and Discussion

Figure 1a shows a region from the ^{13}C, ^{1}H HSQC spectrum of methyl ^{13}C, ^{2}H enriched mouse major urinary protein, containing valine C$^{\gamma1}$-H$^{\gamma1}$ and C$^{\gamma2}$-H$^{\gamma2}$ correlations. Significant overlap is present in the spectrum, arising from the combined effects of interference from resonances from $^{13}CH_3$ isotopomers, together with methyl resonances derived from residues other than valine.

In contrast, an equivalent spectrum recorded on [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine enriched MUP (Figure 1b) is essentially free from resonance overlap, and all 12 valine methyl groups can be observed and assigned from the work of Abbate et al.[33] MUP exhibits significant promiscuity in binding small hydrophobic ligands, and we have chosen to investigate the thermodynamics of binding of 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine on the basis of their global thermodynamics of binding from isothermal titration calorimetry experiments (data not shown). These ligands bind to MUP with K_d's of ~1800 nM and ~300 nM, respectively, at 308 K. Figure 1c shows ^{13}C, ^{1}H HSQC spectra of complexes of these ligands with [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine enriched MUP. Significant shift perturbations are observed only for the correlations from Val 82 C$^{\gamma1}$ and C$^{\gamma2}$, as anticipated since Val 82 is located within the binding pocket of MUP. [37] Stereospecific assignments of Val 82 C$^{\gamma1}$ and C$^{\gamma2}$ in these complexes were confirmed by three-dimensional NOE measurements using ^{15}N, ^{13}C enriched MUP and the relevant unlabeled ligands (data not shown).[38] In both complexes, all correlations are well-resolved, in contrast to complexes with methyl-^{13}C, ^{2}H enriched MUP (data not shown). Under these circumstances, measurement of ^{13}C longitudinal and transverse relaxation rates (R_1 and R_2) can be undertaken in each sample at high sensitivity and with minimal interference due to resonance overlap. Typical relaxation curves are shown in Figure 2. Values of S^2_{axis} were derived from R_1 and R_2 data for each methyl group using the model-free spectral density function[35] given in the General Methods section in the Supporting Information and are listed in Table 1. From these data, it is immediately apparent that there are only minor changes in S^2_{axis} for any of the six valine residues in MUP upon binding of either 2-methoxy-3-isopropylpyrazine or 2-methoxy-3-isobutylpyrazine, most of which are within experimental error. An exception is S^2_{axis} for Val-70 C$^{\gamma}$2, which appears to increase dramatically on binding 2-methoxy-3-isobutylpyrazine. However, this is an anomalous result: Val-70 C$^{\gamma}$1 and C$^{\gamma}$2 possess similar shifts in both complexes and differ by ~3 Hz in the 2-methoxy-3-isobutylpyrazine complex, under which conditions C$^{\gamma}$1 and C$^{\gamma}$2 will be strongly coupled via the two bond homonuclear scalar coupling, which is of similar magnitude. This results in a poor fit of R_2 relaxation data for Val-70 C$^{\gamma}$2 ($\chi^2 = 49.7$).

It is notable that measured S^2_{axis} values for the two methyl groups of certain valine residues are not identical within experimental error. At first sight, this is inconsistent with the requirement for their mobilities to be essentially the same, since they form part of the same isopropyl group. However, this is not necessarily reflected in equivalent S^2_{axis} values: as noted previously,[22,39,40] order parameters of methyl groups from the same isopropyl group may differ if the effective averaging axis for this group makes different angles with the methyl 3-fold axes.

The constant values of S^2_{axis} on complex formation for the methyl groups of Val-82 is unexpected, since this residue is

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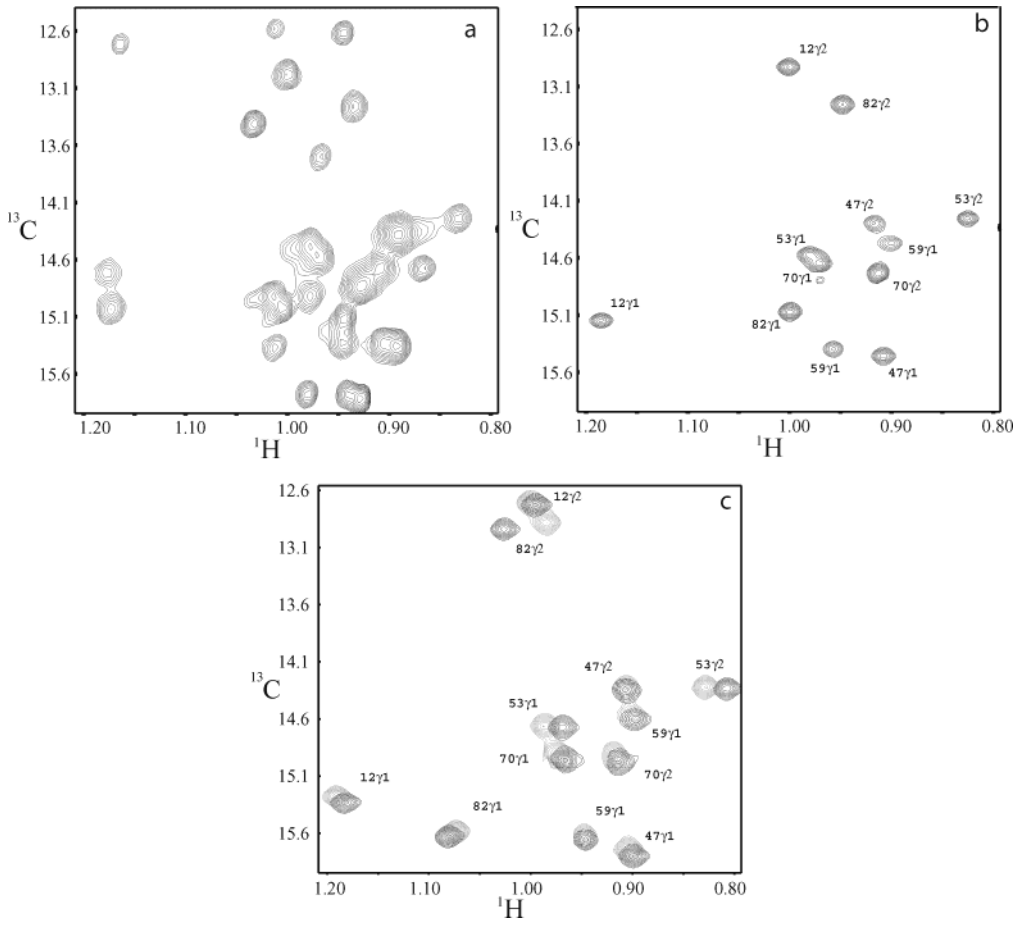

Figure 1. (a) Region of the ^{13}C, ^{1}H HSQC spectrum of methyl-^{13}C, 50% ^{2}H enriched MUP showing valine methyl correlations. (b) Equivalent spectrum of MUP selectively enriched with [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^{2}H_6$] valine. (c) Overlay of regions of the ^{13}C, ^{1}H HSQC spectra of [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^{2}H_6$] valine enriched MUP in complex with 2-methoxy-3-isopropylpyrazine (black correlations) and 2-methoxy-3-isobutylpyrazine (gray correlations).

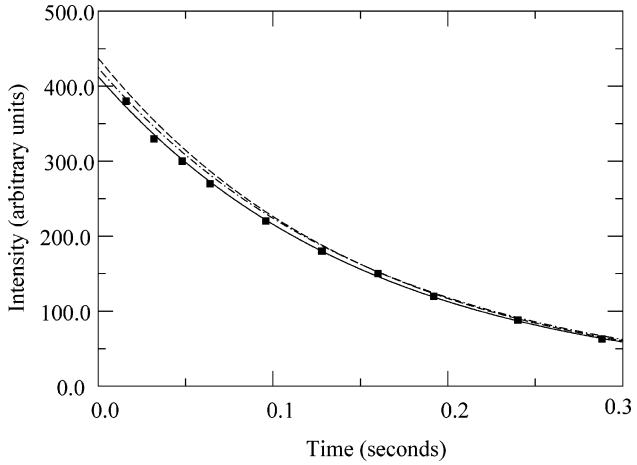

Figure 2. Typical R_2 relaxation curves for Val-12 $C^{\gamma 1}$ obtained from [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^{2}H_6$] valine enriched MUP. Solid line: free protein; dashed line: 2-methoxy-3-isopropylpyrazine complex; dash−dotted line: 2-methoxy-3-isobutylpyrazine complex. For clarity, only the data points corresponding to the free protein are shown. Error bars are smaller than the symbols used for these data points.

Table 1. S^2 Values for Valine Methyl Groups Derived from 600 MHz ^{13}C T_1 and T_2 Measurements on the Major Urinary Protein Selectively Enriched with [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^{2}H_6$] Valine

	ligand		
residue	none S^2_{axis}[a]	2-methoxy-3-isopropyl-pyrazine S^2_{axis}	2-methoxy-3-isobutyl-pyrazine S^2_{axis}
Val-12 $C^{\gamma 1}$	0.88	0.90	0.86
Val-12 $C^{\gamma 2}$	0.77	0.69	0.66
Val-47 $C^{\gamma 1}$	0.91	0.84	0.86
Val-47 $C^{\gamma 2}$	0.86	0.90	0.88
Val-53 $C^{\gamma 1}$	0.74	0.80	0.78
Val-53 $C^{\gamma 2}$	0.90	0.85	0.86
Val-59 $C^{\gamma 1}$	0.73	0.68	0.63
Val-59 $C^{\gamma 2}$	0.98	0.86	0.90
Val-70 $C^{\gamma 1}$	0.78	0.85	0.81
Val-70 $C^{\gamma 2}$	0.77	0.77	(1.03)
Val-82 $C^{\gamma 1}$	0.53	0.50	0.53
Val-82 $C^{\gamma 2}$	0.46	0.48	0.53

[a] The average error in the reported S^2_{axis} values is 0.045 ± 0.009. The bracketed value is anomalous due to strong coupling between Val-70 $C^{\gamma 2}$ and $C^{\gamma 1}$.

located within the binding pocket and is proximal to the bound ligands (R. Bingham and S. E. V. Phillips, unpublished data). Intuitively, the relevant S^2_{axis} values might be expected to increase due to decreased mobility within the binding pocket in the complexes. Since each ligand is protonated, there exists the formal counterintuitive interpretation that additional relaxation pathways offered by ligand protons are offset by an equal and opposite effective contribution due to increased mobility of Val-82 side-chain atoms. In contrast, remaining valine methyl groups are distal to the binding pocket, and ligand protons will make a negligible contribution to relaxation in these cases, since no significant structural changes in MUP are observed in the crystal structures of these complexes (R. Bingham and S. E. V.

Phillips, unpublished data). In the general case where significant structural changes are observed, clearly it would be appropriate to incorporate [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine into a perdeuterated protein, either by use of suitable auxotrophic strains or by overexpression in media containing the relevant ^{2}H amino acids.

A number of researchers have developed approaches for the derivation of thermodynamic parameters from the NMR-derived generalized order parameter.[11−13,15] While all such approaches involve various assumptions such as motional independence of bond vectors and are inherently limited in the motional time scales that can be detected, they are proving to be very valuable as a probe of binding thermodynamics. For example, studies using ^{2}H relaxation methods on calcium-saturated calmodulin in complex with a peptide model of the calmodulin-binding domain have highlighted significant changes in methyl S^2_{axis} values that cannot be predicted in a rational manner: changes to S^2_{axis} values were found at locations both proximal and distal from the Ca^{2+} binding site.[18] In contrast, in the present study we found no changes in valine methyl S^2_{axis} values on ligand binding within experimental error, suggesting that in the present case the entropic contribution to binding is small. Clearly, it is not possible to exclude entropic contributions derived from motions on time scales outside those detectable by ^{13}C relaxation measurements. However, the dominant entropic contribution to binding from MUP dynamics may derive from the backbone, as has been suggested for the complex between MUP and a small-molecule ligand unrelated to the pyrazine derivatives described here.[17]

Conclusions

We have presented a chemical synthetic approach for the preparation of [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine and have demonstrated that incorporation into overexpressed MUP enables measurement of ^{13}C R_1 and R_2 relaxation times for ^{13}-CHD_2 isotopomers that proved impossible in uniformly enriched material. Within experimental error, no changes in S^2_{axis} can be detected for each of the valine methyl groups both in the free protein and in complexes with 2-methoxy-3-isopropylpyrazine and 2-methoxy-3-isobutylpyrazine. This suggests that valine side chains do not contribute significantly to the entropy of binding on time scales detectable by ^{13}C relaxation measurements. In the case of Val-70 C$^\gamma$2, R_2 measurement was hampered in the complex with 2-methoxy-3-isobutylpyrazine by strong coupling between C$^\gamma$1 and C$^\gamma$2 due to near equivalence of chemical shifts. Such complications can be overcome in a modified synthetic strategy that involves selective enrichment of a single methyl group. Alternatively, since the method is highly sensitive, lower (10−20%) levels of ^{13}C enrichment could be utilized. Development of related strategies for the synthesis of the analogous methyl isotopomers of other methyl-containing amino acids is in progress.

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Supporting Information Available: Details of the synthesis of [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine and the expression and purification of [γ^1,γ^2-$^{13}C_2$,α,β,γ^1,γ^1,γ^2,γ^2-$^2H_6$] valine enriched MUP (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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