JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

ARTICLE

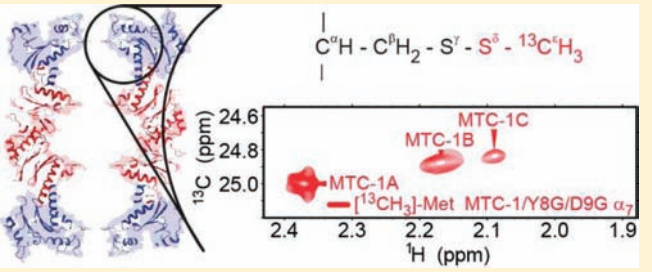
pubs.acs.org/JACS

Site-Directed Methyl Group Labeling as an NMR Probe of Structure and Dynamics in Supramolecular Protein Systems: Applications to the Proteasome and to the ClpP Protease

Tomasz L. Religa, Amy M. Ruschak, Rina Rosenzweig, and Lewis E. Kay*

Departments of Molecular Genetics, Biochemistry and Chemistry, The University of Toronto, Toronto, Ontario M5S 1A8, Canada

ABSTRACT: Methyl groups are powerful reporters of structure, motion, and function in NMR studies of supramolecular protein assemblies. Their utility can be hindered, however, by spectral overlap, difficulties with assignment or lack of probes in biologically important regions of the molecule studied. Here we show that $^{13}CH_3$-S- labeling of Cys side chains using ^{13}C-methyl-methanethiosulfonate (^{13}C-MMTS) (IUPAC name: methylsulfonylsulfanylmethane) provides a convenient probe of molecular structure and dynamics. The methodology is demonstrated with an application focusing on the gating residues of the *Thermoplasma acidophilum* proteasome, where it is shown that the $^{13}CH_3$-S- label reports faithfully on the conformational heterogeneity and dynamics in this region of the complex. A second and related application involves labeling with ^{13}C-MMTS at the N-termini of the subunits comprising the *E. coli* ClpP protease that reveals multiple conformations of gating residues in this complex as well. These N-terminal residues adopt a single conformation upon gate opening.



INTRODUCTION

The development of robust methods for ^{13}C, ^{1}H labeling of Ile, Leu, Val,[1,2] and, more recently, Ala[3,4] and Met[5] methyl groups in otherwise highly deuterated proteins has significantly increased the scope of protein systems that are amenable to detailed quantitative analyses by solution-based NMR methods. Concomitant with the emergence of these labeling approaches has been the use of HMQC-based experiments that exploit a methyl-TROSY effect,[6] enabling studies of supramolecular protein assemblies with molecular weights in the hundreds of kDa. A significant number of applications of this methodology to important systems in biology have now appeared.[4,5,7−15] Critical to their success is that the methyl group probes be localized to regions of interest so that they can be used faithfully as reporters of structure and dynamics, or changes thereof. In addition, the chemical shifts of the methyls must be assigned to specific sites in the primary sequence. In principle, assignments can be obtained using a number of approaches including 'divide and conquer',[5,15] mutagenesis,[5,15] analysis of NOEs[5,15] and pseudocontact shifts[16,17] that are compared with the corresponding values calculated on the basis of crystal structures, or by combined experiment and computation.[18] In practice, generating assignments in very large complexes can be a time-consuming and difficult process.

For many applications the advantages of having as many probes as possible distributed throughout the protein sequence is well worth the effort that must be expended for assignment. In other cases, however, placement of even a single probe in a critical location can be sufficient to answer an important question. For example, studies of the *Thermoplasma acidophilum* proteasome-gating mechanism were hampered by the fact that only a few Ile, Leu, Val methyl groups are in the vicinity of the gates and these gave very weak signals in spectra.[7] In contrast, a Met residue introduced immediately before the wild-type sequence, referred to as Met-1 in what follows, provided critical information (along with the endogenous Met1 and Met6) for establishing that gating is controlled by a stochastic process involving interconversion between a small set of conformations[7] (see below). These experiments were possible only because the number of Met residues in the proteasome is small (five), with three of the five localized to the region of interest, so that well resolved, high sensitivity spectra could be readily obtained. A more general approach, involving site-specific labeling with a methyl-containing moiety distinct in chemical shift from other methyl groups in proteins would be advantageous in extending the utility of methyl labeling yet further.

Cys, with its unique side-chain reactivity and limited occurrence in proteins (1.9%), is a frequent site for selective protein modification.[19,20] Magnetic resonance applications of proteins labeled at Cys positions include elegant nitroxide ESR studies extending over a period of more than two decades.[21] Recently, Cys-attached paramagnetic spin labels have enjoyed a resurgence in NMR applications using either nitroxide[22,23] or metal ion tags.[24,25] One of the simplest modifications of this amino acid is the addition of the CH_3-S- group,[20] which results in formation of S-methylthiocysteine (MTC) (CAS number: 33784-54-2). Due to its simplicity, this moiety has been used extensively in studies of biochemical pathways involving thiol-dependent enzymes.[26,27]

Received: March 11, 2011
Published: May 11, 2011

dx.doi.org/10.1021/ja202259a | *J. Am. Chem. Soc.* 2011, 133, 9063–9068

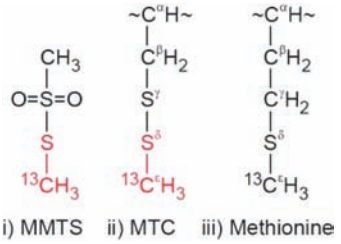


Figure 1. Chemical structures of (i) MMTS, (ii) MTC generated using the MMTS labeling scheme described in the text, and (iii) Met.

This modification is fast and highly specific and can be carried out quantitatively by the addition of a slight molar excess of methyl methanethiosulfonate (MMTS) (IUPAC name: methylsulfonylsulfanylmethane, CAS number: 2949-92-0). In order to explore the utility of this labeling strategy in NMR studies of supramolecular complexes we have applied the methodology to a number of systems that are currently under study in our laboratory. These include (i) the *T. acidophilum* proteasome where ^{13}C-MMTS is added to sites localized to structured regions of the protein or to regions that have been shown previously to adopt multiple conformational states[7] and (ii) the *E. coli* ClpP protease where Met labeling studies proved inconclusive.

RESULTS AND DISCUSSION

The idea of adding $^{13}CH_3$-S- probes specifically to Cys residues in proteins is appealing on a number of levels. First, by using a methyl-containing probe the relaxation properties of the slowly decaying $^{13}C-^{1}H$ multiple-quantum and ^{1}H single-quantum transitions can be exploited to produce spectra of high sensitivity and resolution.[6] Second, as shown in Figure 1, the resultant side chain is structurally very similar to Met, both in total volume and in the number of potential conformers.[28,29] Of all the methyl-containing amino acids Met is the most dynamic,[30] often giving rise to intense $^{13}CH_3$ signals even in studies of very high molecular weight particles.[5,7] An additional advantage is that the spectroscopy of Met-methyl groups is not nearly as adversely affected by μs−ms exchange processes as other methyl probes that are, in general, less averaged by rapid motion, as has been noted previously in our studies of the proteasome.[7,31] Third, unlike the $^{13}CH_3$-Met-labeling approach in which typically fully protonated Met is added to growth media so that protons remain at the C^{β} and C^{γ} positions,[5] the MMTS approach produces 'Met-like' side chains with protonation restricted to the methyl position. Thus, additional protons are not added to the protein, maintaining a very highly deuterated environment in the vicinity of the tag.

An Application to the Proteasome. In order to demonstrate the utility of MMTS labeling we first considered an application to the *T. acidophilum* archaeal proteasome. The proteasome comprises four heptameric rings, forming an $\alpha_7\beta_7\beta_7\alpha_7$ barrel[32] (670 kDa), Figure 2a left, that can be dissected into individual α_7 rings (180 kDa), that in turn consist of α subunits Figure 2a right. These α_7 rings have been shown in a series of previous studies by our group to be excellent model systems of structure and motion in the full proteasome.[7,15] Further, a comparison of spectra of labeled α_7 rings with corresponding data sets recorded on $\alpha_7\beta_7\beta_7\alpha_7$ where only α_7 is labeled establishes that higher-quality spectra are obtained for the individual ring that reflects the smaller size of α_7 in relation to the full proteasome.[7,33] We have therefore chosen to work with α_7 in the present study. A pair of α_7 samples have been produced with MTC placed at position 90 or 95, each of which is located on

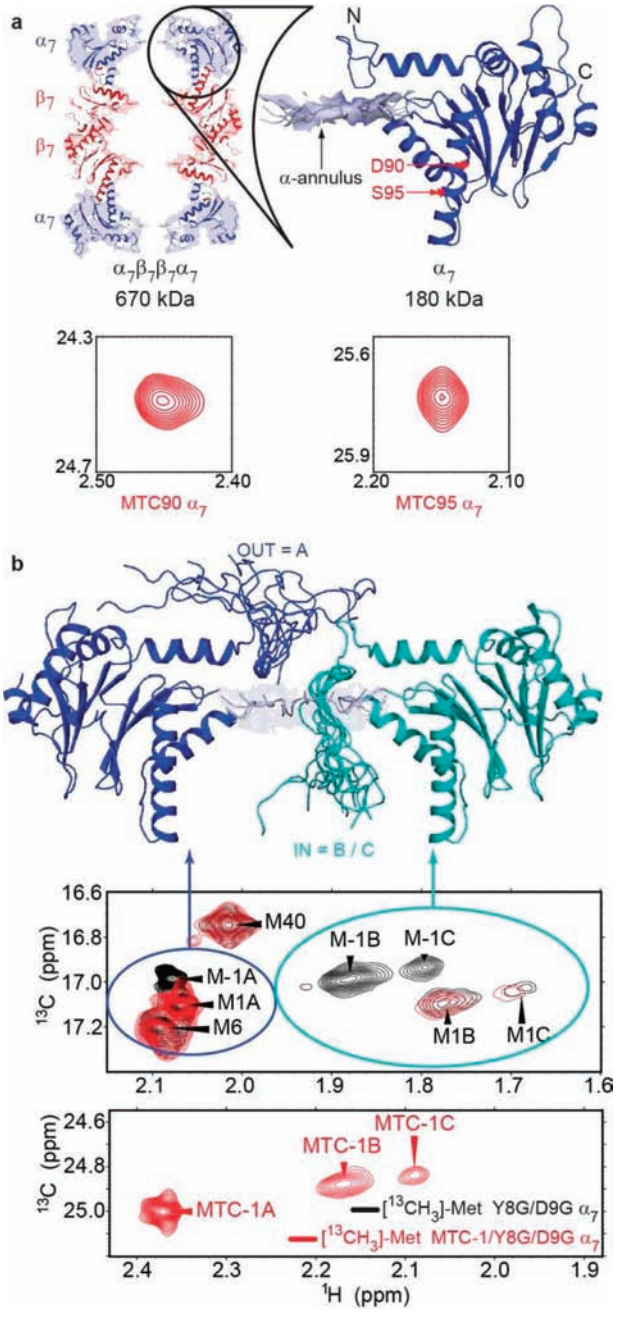


Figure 2. (a) Cross section side view of the structure of the full 20S proteasome, comprised of an $\alpha_7\beta_7\beta_7\alpha_7$ four ring structure[34] (α_7-blue, β_7-red, left) and the structure of α_7 (right, 1 of the 7 α-subunits is highlighted, along with the α-annulus ring that comprises residues from all 7 subunits). $^{1}H-^{13}C$ HMQC spectra of U-^{2}H, MTC90 α_7 and U-^{2}H, MTC95 α_7, recorded at 25 °C, 800 MHz. Single peaks are observed for the MTC methyl probes. (b) $^{1}H-^{13}C$ HMQC spectra of U-^{2}H,Met-[$^{13}CH_3$] Y8G/D9G α_7 (black) and U-^{2}H, Met-[$^{13}CH_3$] MTC-1/Y8G/D9G α_7 (red). Note that the M-1 peaks are eliminated in the spectrum of U-^{2}H, Met-[$^{13}CH_3$] MTC-1/Y8G/D9G α_7, as expected, replaced by correlations that are shifted to $(\omega^{13}C,\omega^{1}H) \approx$ (25 ppm, 2.3 ppm). Also shown are two of the seven subunits of α_7 with the N-terminal residues in the OUT or IN positions, giving rise to separate sets of resonances indicated by 'A' and 'B'/'C', respectively, in the figure. Ten termini are shown in each position, corresponding to an ensemble of structures calculated previously.[7].

dx.doi.org/10.1021/ja202259a |J. Am. Chem. Soc. 2011, 133, 9063–9068

helix H1, a well-structured region of the molecule, Figure 2a. As described in Materials and Methods this is achieved by substituting Cys for the wild-type residues at these sites and then reacting with ^{13}C-MMTS. High-quality data sets were obtained for each α_7 sample (25 °C, 800 MHz), Figure 2a, with the resonance positions of each $^{13}CH_3$-S moiety, $(\omega^{13}C,\omega^{1}H) \approx$ (25 ppm, 2.3 ppm), localized to a region of the $^{13}C,^{1}H$ correlation map that is typically unobstructed by protein resonances. In principle, the MMTS-labeling strategy can therefore be used in studies of proteins that are fully protonated without complications from natural abundance peaks. In practice, however, we have found the losses in sensitivity and resolution to be prohibitive in cases where fully protonated samples were utilized, due to increased relaxation rates of methyl probe coherences. For example, in a comparison of spectra recorded on U-^{2}H,MTC95 α_7 (*i.e.*, α_7 with MTC at position 95) and fully protonated MTC95 α_7 the signal-to-noise of the MTC peak was decreased by a factor of 4 in the protonated sample (both samples were of the same concentration). In the case of the corresponding samples labeled with MTC at position 90 the decrease was 2-fold. It is noteworthy that positions 95 and 90 have solvent-accessible surface areas of 4% and 30%, respectively, so that the losses in sensitivity are related to proton density, as expected. The importance of deuteration is thus clear in applications involving high molecular weight systems where sensitivity and resolution are almost always limiting factors.

In a previous study we have used Met-$^{13}CH_3$- groups in the α-rings to elucidate the structure, dynamics, and mechanism of proteasome gating,[7] the process by which entry of substrate into the catalytic chamber of the proteasome is regulated. As a 'test' of the labeling we wish to establish that similar results are obtained using the MTC probe in place of Met. As a reference, the methyl-TROSY spectrum of highly deuterated, Met-[$^{13}CH_3$] Y8G/D9G α_7 (single ring) is shown in Figure 2b (black, 25 °C, 800 MHz), displaying multiple resonances for the N-terminal Met residues (M-1, M1) due to a slow exchange process that places the gate either outside (labeled state A in Figure 2b) or inside (states B/C) the antechamber.[7] Note that the N-terminus of the α-subunit includes the cloning artifact GAMG, so that the first Met residue, at position −1, is not a part of the wild-type sequence. A second sample of Y8G/D9G α_7 was produced where Met-1 was replaced by Cys that was then reacted with ^{13}C-MMTS label (all other Met were retained and are labeled as $^{13}C^{\varepsilon}H_3$). The spectrum of this molecule (denoted as Met-[$^{13}CH_3$],MTC-1/Y8G/D9G α_7) is shown in Figure 2b in red. Notably, all of the correlations from the three states of M1 and from M6 and M40 are superimposed on those from the Met-[$^{13}CH_3$] sample indicating that the MTC at position −1 has not perturbed the chemical environments of the remaining Met residues. As expected the M-1 correlations are missing, replaced by cross-peaks from MTC-1 at a position downfield in both the ^{13}C and ^{1}H dimensions of the HMQC data set, Figure 2b. Despite the different chemical shifts, the pattern of the MTC-1 correlations reproduced well those from M-1 and the populations of the different states based on MTC-1 peak intensities, 60:29:11% (A:B:C, error 2%) at 25 °C and 56:27:17 at 40 °C were very similar to what was observed previously at 50 °C using M-1 cross-peaks (55%A, 45%B+C) (ref 7). Finally, it is worth mentioning that the correlation between peak positions for the Met and MTC methyl probes observed at the −1 gating position (that is, site A is downfield of B that in turn is downfield of C, in both ^{1}H and ^{13}C dimensions for both probes)

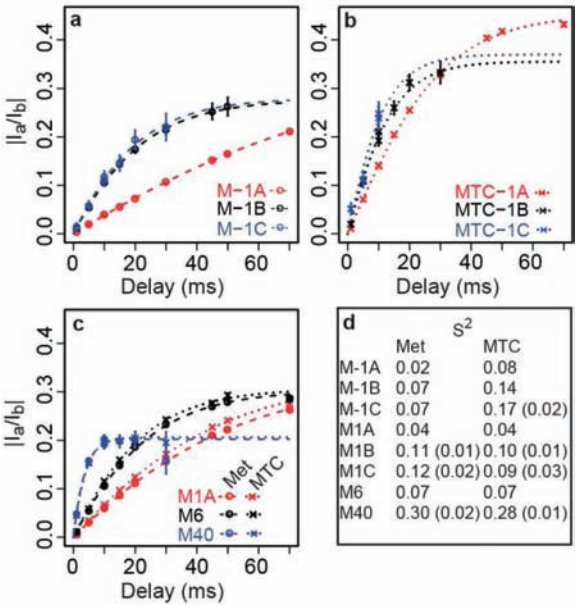


Figure 3. Values of $|I_a/I_b|$ vs relaxation delay plotted for M-1 of U-^{2}H, Met-[$^{13}CH_3$] Y8G/D9G α_7 (a) with the corresponding ratios for the −1 position of U-^{2}H,Met-[$^{13}CH_3$] MTC-1/Y8G/D9G α_7 (referred to as MTC-1A, MTC-1B, MTC-1C) in (b). (c), as in (a) and (b) but for M1 (A peak), M6 and M40. Essentially identical $|I_a/I_b|$ vs relaxation delay profiles are obtained for the two samples (circles, measured on Met-[$^{13}CH_3$] Y8G/D9G α_7; crosses, measured on Met-[$^{13}CH_3$] MTC-1/Y8G/D9G α_7) indicating that the dynamics at these positions are not influenced by the Met to MTC substitution at position −1. (d) Table of S^2 values quantifying the amplitudes of Met or MTC $C^{\varepsilon}-S^{\delta}$ bond vector motions (25 °C). Errors ≥0.01 are shown in parentheses.

is also noted in a comparison of M90 α_7 and MTC90 α_7 spectra or the corresponding M95/MTC95 α_7 data sets. This provides a further indication as to the similarities of the Met and MTC methyl probes.

It is of interest to compare the ps−ns dynamics of the Met and MTC side chains that differ only in the replacement of a $C^{\gamma}H_2$ group by S^{γ} (Figure 1), especially since side-chain dynamics can play an important role in providing high-quality data sets. Structurally, the $C^{\beta}-S^{\gamma}$ and $S^{\gamma}-S^{\delta}$ bonds of MTC are longer than the corresponding $C^{\beta}-C^{\gamma}$ and $C^{\gamma}-S^{\delta}$ bonds of Met, and there is a more restricted set of rotamers for the disulfide relative to a C−C bond.[35,36] It might be expected therefore that the amplitude of the motion of the methyl group of MTC would be more restricted than for Met. We have quantified squared order parameters, S^2, for the $^{13}C^{\varepsilon}-S^{\delta}$ bonds of Met and MTC at position −1 of U-^{2}H,Met-[$^{13}CH_3$] Y8G/D9G α_7 and U-^{2}H, Met-[$^{13}CH_3$] MTC-1/Y8G/D9G α_7, respectively, using an approach that measures the time-dependencies of sums (I_b) and differences (I_a) of magnetization derived from methyl ^{1}H single quantum transitions.[37] Values of $|I_a/I_b|$ vs relaxation delay are plotted for M-1 of U-^{2}H,Met-[$^{13}CH_3$] Y8G/D9G α_7 in Figure 3a with the corresponding ratios for the −1 position of U-^{2}H,Met-[$^{13}CH_3$] MTC-1/Y8G/D9G α_7 (containing the MTC label at −1 and referred to as MTC-1A, MTC-1B, MTC-1C) shown in Figure 3b. Noting that as the mobility of the methyl probe increases the build up of $|I_a/I_b|$ becomes slower,[37] it is clear that the Met-methyl group is more mobile than the methyl of the MTC moiety. The two compared proteins differ only in the modification at position −1 (see above); all the

dx.doi.org/10.1021/ja202259a |*J. Am. Chem. Soc.* 2011, 133, 9063–9068

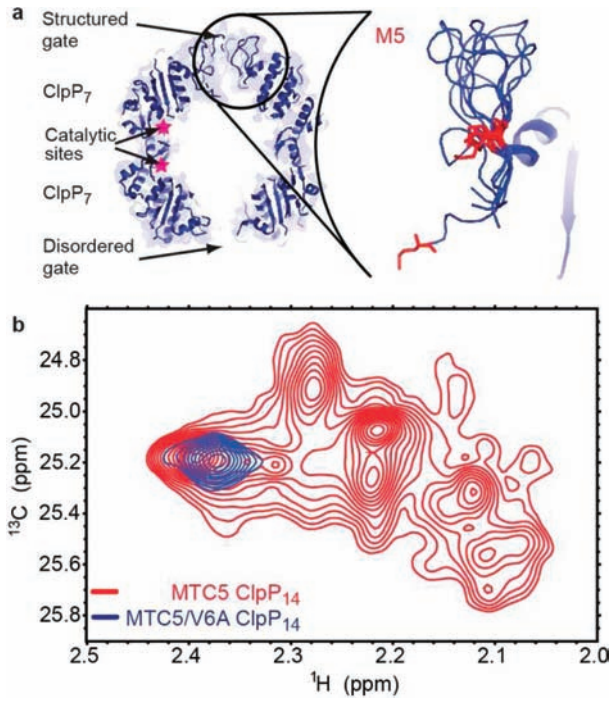


Figure 4. (a) Structure of *E.coli* ClpP (1YG6) (ref 42), where the N-terminal gating residues in the top ring of the two-ring structure crystallized in multiple conformations, while electron density was not observed for the N-termini in the bottom ring (top, left). Residues 20−193 of the seven subunits from the top ring have been superimposed, with the location of M5 shown in red (top, right). (b) $^{1}H-^{13}C$ HMQC spectrum of MTC5 ClpP (red) and MTC5/V6A ClpP (blue), 37 °C, 600 MHz.

corresponding Met groups in both molecules have nearly identical $|I_a/I_b|$ profiles, Figure 3c (compare circles and crosses), indicating that MTC at position −1 does not interfere with the motional properties of Met methyl groups at other sites. In Figure 3d S^2 values extracted from fits of $|I_a/I_b|$ curves are shown (25 °C). The mobility of the MTC methyl, although reduced relative to the corresponding Met methyl group, is still very high with S^2 values under 0.2. It may well be that in a more restrictive environment than that of a disordered terminus the differences in ps-ns mobilities between Met and MTC would be even less.

An application to the *E. coli* ClpP Protease. The proteasome is one example of a gated self-compartmentalized protease,[38,39] where access to the active sites that are sequestered in the lumen of the barrel-shaped chamber is controlled by the diameter of a narrow axial channel, referred to as an α-annulus (Figure 2a). Intracellular protein degradation in bacteria, which mostly lack the proteasome,[39] is relegated to the 300 kDa ClpP protease, formed by the stacking of two homoheptameric rings[40,41] (Figure 4a, left). Here, too, N-terminal residues control access to the lumen of the protease and, consequently, to the active sites. These N-terminal residues are dynamic; they have been shown to adopt multiple conformations in crystals[42] (Figure 4a, right) and in many cases little or no density is observed for them.[43] In the *E. coli* version studied here the presence of ten Met residues complicates using methyl groups from this amino acid as probes. The situation using Met residues is further complicated by the fact that well over 20 correlations are observed in $^{13}C,^{1}H$ correlation maps recorded of $^{13}CH_3$-Met-labeled ClpP protease so that assignment of cross-peaks to specific sites in the protein is difficult. As a result we have made use of MMTS to specifically label different gate residues in the protein.

Figure 4b (red) shows the $^{13}C,^{1}H$ HMQC spectrum of ClpP prepared with label at position 5, U-^{2}H,MTC5 $ClpP_{14}$ (37 °C, 600 MHz, red), and the large number of peaks (over 10) establish that there are numerous conformations for the gate at this site. By contrast, when label was placed at the same position in a V6A mutant of $ClpP_{14}$ (MTC5/V6A $ClpP_{14}$), a spectrum with only a single correlation was observed (Figure 4b, blue). It is known that the hydrolysis rates of substrate peptides increase for the V6A mutant indicating that this mutation generates an "open" state of the gate[42] that is substrate accessible. The observation of only a single peak in this case is consistent with a single conformation (blue peak) that corresponds to one of the states that is observed in the wild-type enzyme (note the proximity of the blue peak to one of the correlations in the red spectrum). If the gate were exchanging rapidly between all of the conformations that are sampled by the wild-type enzyme then a single peak would be observed as well. However, in this case the position of such a correlation would be derived from a weighted average of chemical shifts of all of the 'red' peaks in Figure 4b placing it in a different location to what is observed. It is likely, therefore, that the intense peak at $(\omega^{13}C,\omega^{1}H)$=(25.2 ppm, 2.4 ppm) in the spectrum of U-^{2}H,MTC5 $ClpP_{14}$ corresponds to the "open" state of the gating residues with the additional peaks derived from multiple conformers associated with the "closed" states of these gating termini, although this remains to be investigated more fully. Further, this result suggests that the N-termini of the ClpP protease might exist in both "open" and "closed" conformations, as has been observed for the proteasome[7] (Figure 2b). Analysis of the range of different structures that are sampled by the gate, the dynamics of these residues and the lifetimes of the different conformers will be the subject of future studies. However, the complexity of spectra obtained from only a single $^{13}CH_3$-group in the case of MTC5 $ClpP_{14}$ suggests strongly that this analysis will only be possible using a site-specific label of the sort we are advocating here.

In summary, the MMTS reagent provides a means for the straightforward introduction of a $^{13}CH_3$-S- group at reactive Cys positions to form MTC that can be used as a highly specific probe of protein structure, binding, or dynamics in supramolecular systems. Additional protons, beyond those of the methyl group, are not introduced by this labeling scheme so that high-quality data sets can be recorded. We have shown that the MTC label is a sensitive reporter of protein conformation for both the proteasome and the ClpP protease. MTC will serve a particularly important role for rapidly probing a particular region of structure without the requirement for resonance assignments and with the high sensitivity and resolution that can be achieved using methyl group reporters.

MATERIALS AND METHODS

Protein Expression and Purification. Samples of highly deuterated Met-[$^{13}CH_3$] Y8G/D9G α_7, D90C α_7, S95C α_7, and M-1C/Y8G/D9G α_7 were expressed and purified as described previously.[7,15,33] Methyl groups of Met were labeled with $^{13}CH_3$ by supplementing the M9 D_2O-based growth media with 100 mg/L of $^{13}CH_3$ (otherwise ^{1}H)-labeled Met (Sigma-Aldrich) one hour prior to protein induction. Cys mutants of α_7 were reacted with ^{13}C-MMTS (Sigma-Aldrich) to generate MTC at the site of mutation, as described below. The single Cys present in the wild-type α-subunit (Cys 151) does not react with

MMTS, as established by ESI-MS. Samples comprising between 0.8 and 1.5 mM protein (in monomer of α; 0.11−0.21 mM in α_7 particle) were prepared in 100% D_2O, 25 mM potassium phosphate, pH 6.8, 50 mM NaCl, 1 mM EDTA, 0.03% NaN_3 and used for all experiments. All HMQC spectra were acquired at 25 °C on a Varian 800 MHz spectrometer in 1−2 h of measurement time/spectrum.

E.coli ClpP was expressed from a modified pET9d kanamycin plasmid (NEB) and purified under native conditions using Ni and gel filtration columns. A number of gene modifications were performed using the QuikChange protocol (Stratagene), including removal of the prosequence, insertion of an S97A mutation that disables proteolysis activity and addition of 6 His residues to the C-terminus. *E. coli* Codon+ RIPL cells (Stratagene) were transformed with the pET9d plasmid containing the desired ClpP mutant. The cells were grown at 37 °C in M9 D_2O media supplemented with 2 g/L of ^2H glucose until an $OD_{600} \approx 1$ was reached, when expression was induced by addition of 1 mM IPTG and allowed to proceed overnight. The bacteria were harvested and resuspended in Ni-A buffer (20 mM imidazole, 50 mM potassium phosphate, 200 mM NaCl, pH 8.0, 0.03% NaN_3, protease inhibitor tablets (Roche)). The cells were lysed by sonication and spun down at 45000*g* for 30 min. The filtered supernatant was applied to a column with Ni Sepharose beads (GE Healthcare), equilibrated and washed with Ni-A buffer. ClpP was eluted using Ni−B buffer (500 mM imidazole, 50 mM potassium phosphate, 200 mM NaCl, pH 8.0, 0.03% NaN_3). After concentrating on an Amicon Ultra-15 30K molecular weight cutoff (MWCO) filter (Millipore), the protein was further purified on a HiLoad 16/60 Superdex 200 pg gel filtration column (GE Healthcare), equilibrated with 50 mM potassium phosphate, 100 mM NaCl, pH 7.5, 0.03% NaN_3, where it eluted at ∼71 mL. The purity was confirmed by SDS-PAGE and ESI-MS. No intrinsic *E. coli* ClpP was observed, likely due to high overexpression levels from the T7-based promoter of the pET9d vector and His-tag based purification methodology. The M5C ClpP mutant was reacted with MMTS as described below. As for the proteasome samples, reactions involving native Cys sites (Cys 91 and Cys 113) were not observed by MS or gel electrophoresis (see below). ClpP NMR samples were prepared in 100% D_2O, 10 mM potassium phosphate, pH 8.0, 0.03% NaN_3, 0.5 mM EDTA. All HMQC spectra were acquired at 37 °C on a Varian 600 MHz spectrometer.

$^{13}CH_3$-S- Labeling with MMTS. Purified Cys mutants of ClpP and α7, stored with 5 mM DTT, were buffer exchanged with an Amicon Ultra-15 30K MWCO (Millipore) into 1 mL of ice-cold, degassed 50 mM potassium phosphate, pH 7.5, 0.03% NaN_3, 1 mM EDTA. A DMSO stock solution of 100 mM ^{13}C-MMTS, stored at −20 °C, was added in 50% molar excess to a solution of the protein and allowed to react overnight at 4 °C. The reactions were terminated by buffer exchange into the appropriate NMR buffer. All samples were submitted for ESI-MS to verify the incorporation of the $^{13}CH_3$-S- group and to establish that other Cys positions in the proteins were not labeled. SDS-PAGE gel electrophoresis was performed to ensure the absence of disulfide bonds between α subunits. In general, MMTS labeling reactions are very robust and likely require shorter times than those used here, without the need to go to lower temperatures.[20] The applications involving labeling of gating residues considered in this study are particularly demanding because there are seven N-termini on each α_7 or ClpP ring, and these termini are proximal. When the protein of interest is to be reacted with MMTS, reducing agents such as DTT must first be removed, and oxidation of the reactive Cys residues in the termini is a distinct possibility. With this in mind the labeling reactions were done at low temperatures, under conditions where the concentrations of disulfide-catalyzing metal ions were low (addition of EDTA) and degassed buffers were used.

A potential concern in using MMTS in studies of structures like that of the gate moiety of the proteasome, in which large numbers of labels are positioned on flexible segments of polypeptide that can potentially interact, is that label can be lost over time.[7] Samples prepared as described here were shown by mass spectrometry (MS) to be labeled stoichiometrically by MMTS (1 MTC moiety for each α-subunit), and SDS gel electrophoresis confirmed the absence of any intersubunit disulfide linkages. Furthermore, labeling did not occur in the absence of the M-1C mutation. However, in NMR experiments performed overnight at 40 °C a drop in signal intensity from the MTC-1 moiety of approximately 10% was observed. This was coupled with the appearance of an unlabeled subunit (α_1) and a dimer peak in MS spectra (α_2) with a mass consistent with the removal of the MMTS label for both α-subunits. Formation of an α-S−S-α structure (*i.e.*, a disulfide cross-linked dimer) suggests a thiol-disulfide exchange reaction that is promoted by high temperatures and, of course, by the proximity of the S-methanethiol cysteine residues on each of the seven α-subunits of the α_7 ring. Dimer formation and loss of the methanethiol group is very unlikely to be problematic in most applications, where the local concentration of thiol groups will be significantly lower than in the proteasome gate region. Indeed, in studies where MTSL spin labels (same chemistry as MMTS labels) were placed at positions distinct from the gates in the proteasome, no changes in spectra were noted after at least several days of measurement at 50 °C (ref 7). Of course, rearrangements of the sort described here could be completely avoided by using an irreversible labeling reagent, such as ^{13}C-methyl-labeled *N*-ethylmaleimide,[44] although the probe in this case is quite sizable. By working at 25 °C, intensity changes of <5% were observed over the time course of the proteasome experiments reported here (several days).

Extraction of S^2 Values Reporting on the Amplitudes of ps−ns Time-Scale Motions. S^2_{axis} values quantifying the amplitudes of Met or MTC $C^{\varepsilon}-S^{\delta}$ bond vector motions were measured (25 °C)and analyzed as described by Tugarinov et al.[37] using an approach in which the time dependencies of the sums (I_b) and differences (I_a) of methyl ^1H single-quantum transitions are quantified. Values of I_a and I_b were measured for time points of (in this order): 0.001, 0.01, 0.07, 0.005, 0.05, 0.015, 0.03, 0.02, 0.01, and 0.045 s. The profiles I_a/I_b were fitted to

$$I_a/I_b = \frac{-0.5\eta \tanh\left(\sqrt{\eta^2+\delta^2}T\right)}{\sqrt{\eta^2+\delta^2} - \delta \tanh\left(\sqrt{\eta^2+\delta^2}T\right)}$$

where δ is a parameter that is related to the ^1H spin density around the methyl group in question and

$$\eta \approx \frac{9}{10}\left[P_2(\cos\theta_{\mathrm{axis,HH}})\right]^2 \frac{S^2_{\mathrm{axis}}\gamma_H^4\hbar^2\tau_c}{r_{HH}^6}$$

with τ_c the tumbling time of the assumed isotropically rotating particle, γ_H is the gyromagnetic ratio of a proton spin, r_{HH} is the distance between pairs of methyl protons (1.813 Å), $P_2(x) = (1/2)(3x^2-1)$, and $\theta_{\mathrm{axis,HH}}$ (90°) is the angle between the methyl three-fold axis and a vector that connects a pair of methyl ^1H nuclei. A value of τ_c = 102 ns has been used in the present analysis, one-half the value of the $\alpha_7\alpha_7$ particle that was measured at 50 °C (125 ns) (ref 15), corrected for the increase in viscosity of the buffer at 25 °C. Errors in I_a, I_b values were estimated by measuring a duplicate set of spectra at a time point of 0.01 s.

AUTHOR INFORMATION

Corresponding Author
kay@pound.med.utoronto.ca

ACKNOWLEDGMENT

T.L.R acknowledges the European Molecular Biology Organization (EMBO) and the Canadian Institutes of Health Research (CHIR) for postdoctoral fellowships. R.R is funded

with an EMBO fellowship. Prof. Julie Forman-Kay and Dr. Ranjith Muhandiram are thanked for providing laboratory space and for NMR support, respectively. Elisa Leung and Prof. Walid A. Houry are acknowledged for providing the ClpP plasmids used in this study. This work was supported by a grant from the CIHR. L.E.K holds a Canada Research Chair in Biochemistry.

REFERENCES

(1) Tugarinov, V.; Kay, L. E. *J. Biomol. NMR* **2004**, *28*, 165.
(2) Tugarinov, V.; Kay, L. E. *ChemBioChem* **2005**, *6*, 1567.
(3) Ayala, I.; Sounier, R.; Use, N.; Gans, P.; Boisbouvier, J. *J. Biomol. NMR* **2009**, *43*, 111.
(4) Isaacson, R. L.; Simpson, P. J.; Liu, M.; Cota, E.; Zhang, X.; Freemont, P.; Matthews, S. *J. Am. Chem. Soc.* **2007**, *129*, 15428.
(5) Gelis, I.; Bonvin, A. M.; Keramisanou, D.; Koukaki, M.; Gouridis, G.; Karamanou, S.; Economou, A.; Kalodimos, C. G. *Cell* **2007**, *131*, 756.
(6) Tugarinov, V.; Hwang, P. M.; Ollerenshaw, J. E.; Kay, L. E. *J. Am. Chem. Soc.* **2003**, *125*, 10420.
(7) Religa, T. L.; Sprangers, R.; Kay, L. E. *Science* **2010**, *328*, 98.
(8) Ruschak, A. M.; Religa, T. L.; Breuer, S.; Witt, S.; Kay, L. E. *Nature* **2010**, *467*, 868.
(9) Velyvis, A.; Schachman, H. K.; Kay, L. E. *J. Mol. Biol.* **2009**, *387*, 540.
(10) Amero, C.; Schanda, P.; Dura, M. A.; Ayala, I.; Marion, D.; Franzetti, B.; Brutscher, B.; Boisbouvier, J. *J. Am. Chem. Soc.* **2009**, *131*, 3448.
(11) Velyvis, A.; Yang, Y. R.; Schachman, H. K.; Kay, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 8815.
(12) Kreishman-Deitrick, M.; Goley, E. D.; Burdine, L.; Denison, C.; Egile, C.; Li, R.; Murali, N.; Kodadek, T. J.; Welch, M. D.; Rosen, M. K. *Biochemistry* **2005**, *44*, 15247.
(13) Sprangers, R.; Gribun, A.; Hwang, P. M.; Houry, W. A.; Kay, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16678.
(14) Hamel, D. J.; Dahlquist, F. W. *J. Am. Chem. Soc.* **2005**, *127*, 9676.
(15) Sprangers, R.; Kay, L. E. *Nature* **2007**, *445*, 618.
(16) John, M.; Schmitz, C.; Park, A. Y.; Dixon, N. E.; Huber, T.; Otting, G. *J. Am. Chem. Soc.* **2007**, *129*, 13749.
(17) Velyvis, A.; Schachman, H. K.; Kay, L. E. *J. Am. Chem. Soc.* **2009**, *131*, 16534.
(18) Xu, Y.; Liu, M.; Simpson, P. J.; Isaacson, R.; Cota, E.; Marchant, J.; Yang, D.; Zhang, X.; Freemont, P.; Matthews, S. *J. Am. Chem. Soc.* **2009**, *131*, 9480.
(19) Chalker, J. M.; Bernardes, G. J.; Lin, Y. A.; Davis, B. G. *Chem. Asian J.* **2009**, *4*, 630.
(20) Kenyon, G. L.; Bruice, T. W. *Methods Enzymol.* **1977**, *47*, 407.
(21) Todd, A. P.; Cong, J.; Levinthal, F.; Levinthal, C.; Hubbell, W. L. *Proteins* **1989**, *6*, 294.
(22) Allison, J. R.; Varnai, P.; Dobson, C. M.; Vendruscolo, M. *J. Am. Chem. Soc.* **2009**, *131*, 18314.
(23) Clore, G. M. *Mol. Biosyst.* **2008**, *4*, 1058.
(24) Otting, G. *Annu. Rev. Biophys.* **2010**, *39*, 387.
(25) Keizers, P. H.; Ubbink, M. *Prog. Nucl. Magn. Reson. Spectrosc.* **2011**, *58*, 88.
(26) Smith, D. J.; Kenyon, G. L. *J. Biol. Chem.* **1974**, *249*, 3317.
(27) Smith, D. J.; Maggio, E. T.; Kenyon, G. L. *Biochemistry* **1975**, *14*, 766.
(28) Wynn, R.; Richards, F. M. *Protein Sci.* **1993**, *2*, 395.
(29) Auld, D. S.; Young, G. B.; Saunders, A. J.; Doyle, D. F.; Betz, S. F.; Pielak, G. J. *Protein Sci.* **1993**, *2*, 2187.
(30) Mittermaier, A.; Kay, L. E.; Forman-Kay, J. D. *J. Biomol. NMR* **1999**, *13*, 181.
(31) Baldwin, A. J.; Religa, T. L.; Hansen, D. F.; Bouvignies, G.; Kay, L. E. *J. Am. Chem. Soc.* **2010**, *132*, 10992.
(32) Lowe, J.; Stock, D.; Jap, B.; Zwickl, P.; Baumeister, W.; Huber, R. *Science* **1995**, *268*, 533.
(33) Sprangers, R.; Li, X.; Mao, X.; Rubinstein, J. L.; Schimmer, A. D.; Kay, L. E. *Biochemistry* **2008**, *47*, 6727.
(34) Forster, A.; Masters, E. I.; Whitby, F. G.; Robinson, H.; Hill, C. P. *Mol. Cell* **2005**, *18*, 589.
(35) Hazes, B.; Dijkstra, B. W. *Protein Eng.* **1988**, *2*, 119.
(36) Richardson, J. S. *Adv. Protein Chem.* **1981**, *34*, 167.
(37) Tugarinov, V.; Sprangers, R.; Kay, L. E. *J. Am. Chem. Soc.* **2007**, *129*, 1743.
(38) Baumeister, W.; Walz, J.; Zuhl, F.; Seemuller, E. *Cell* **1998**, *92*, 367.
(39) Groll, M.; Bochtler, M.; Brandstetter, H.; Clausen, T.; Huber, R. *ChemBioChem* **2005**, *6*, 222.
(40) Yu, A. Y.; Houry, W. A. *FEBS Lett.* **2007**, *581*, 3749.
(41) Kimber, M. S.; Yu, A. Y.; Borg, M.; Leung, E.; Chan, H. S.; Houry, W. A. *Structure* **2010**, *18*, 798.
(42) Bewley, M. C.; Graziano, V.; Griffin, K.; Flanagan, J. M. *J. Struct. Biol.* **2006**, *153*, 113.
(43) Wang, J.; Hartling, J. A.; Flanagan, J. M. *Cell* **1997**, *91*, 447.
(44) Partis, M. D.; Griffiths, D. G.; Roberts, G. C.; Beechey, R. B. *J. Protein Chem.* **1983**, *2*, 263.