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Differences in Lysine pKa Values May Be Used to Improve NMR Signal Dispersion in Reductively Methylated Proteins

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Summary

Reductive methylation of lysine residues in proteins offers a way to introduce ¹³C methyl groups into otherwise unlabeled molecules. The ¹³C methyl groups on lysines possess favorable relaxation properties that allow highly sensitive NMR signal detection. One of the major limitations in the use of reductive methylation in NMR is the signal overlap of ¹³C methyl groups in NMR spectra. Here we show that the uniform influence of the solvent on chemical shifts of exposed lysine methyl groups could be overcome by adjusting the pH of the buffering solution closer to the pKa of lysine side chains. Under these conditions, due to variable pKa values of individual lysine side chains in the protein of interest different levels of lysine protonation are observed. These differences are reflected in the chemical shift differences of methyl groups in reductively methylated lysines. We show that this approach is successful in four different proteins including Ca²⁺-bound Calmodulin, Lysozyme, Ca²⁺-bound Troponin C, and Glutathione S-Transferase. In all cases significant improvement in NMR spectral resolution of methyl signals in reductively methylated proteins was obtained. The increased spectral resolution helps with more precise characterization of protein structural rearrangements caused by ligand binding as shown by studying binding of Calmodulin antagonist trifluoperazine to Calmodulin. Thus, this approach may be used to increase resolution in NMR spectra of ¹³C methyl groups on lysine residues in reductively methylated proteins that enhances the accuracy of protein structural assessment.

Keywords

reductive methylation; NMR; lysine; pKa

Introduction

Reductive methylation is a chemical modification that allows specific introduction of ¹³C-enriched methyl groups into lysine residues in otherwise unlabeled proteins (Means & Feeney 1968; Rayment 1997). Methylation of lysines does not significantly perturb protein structure and does not induce spurious protein-protein interactions (Gerken et al. 1982; Kurinov et al. 2000; Rayment 1997; Rypniewski et al. 1993; Walter et al. 2006). The methylated lysines retain their positive charge and are able to participate in intra- and intermolecular binding events. While mostly unused in current NMR experimental schemes, reductive methylation offers several advantages to NMR spectroscopy. For example, because of its post-translational nature

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Supplemental Material: MALDI-TOF mass spectrometry data for methylated and unmethylated GST.

reductive methylation allows the use of proteins purified from organisms for which there are no available isotope enrichment protocols. Furthermore, due to reduced order parameters for lysine side-chains, the ^{13}C methyl groups on lysines possess favorable relaxation properties allowing sensitive NMR signal detection for large molecular weight proteins (Abraham et al. 2008). Methyl groups on lysine residues may be used to detect conformational rearrangements due to protein-ligand interactions even when lysines are not directly involved in binding interfaces (Abraham et al. 2008). Determination of lysine pKa values using NMR and reductive methylation has also been demonstrated (Zhang & Vogel 1993).

Despite the advantages offered by reductive methylation to NMR spectroscopy, there are several limitations that hinder a wider use of this labeling technique. Some proteins may be susceptible to inactivation by reductive methylation (Ganzhorn et al. 1996). Apart from point-mutagenesis and the recently introduced matching of the rates of methylation measured by NMR and mass-spectrometry there are no straight-forward methods for resonance assignments of reductively methylated proteins (Macnaughtan et al. 2005). Finally, the lack of signal dispersion in NMR spectra of reductively methylated proteins significantly limits the number of probes available for structural assessment (Abraham et al. 2008). The latter limitation stems from the fact that most lysines are solvent exposed and face a very similar electronic environment. Limited NMR chemical shift dispersion at lysine side chain positions has been previously demonstrated (Gao et al. 2006; Iwahara et al. 2007).

Here we address the problem of limited signal dispersion in NMR spectra of reductively methylated proteins. We postulate that the amino acids proximal to the lysines in the protein provide small but unique contributions to the chemical environment for individual lysine side chains. These unique contributions are evidenced by the fact that the pKa values for individual lysines in protein molecules vary significantly. For example, in calcium binding proteins Calmodulin (Zhang & Vogel 1993) and Calbindin D9k (Kesvatera et al. 1996) lysine pKa values vary from 9.3 to 13.2. Reductive methylation has been reported to lower the apparent pKa values for lysine residues (Andre et al. 2007). At pH values close to 7.0 most lysines are protonated and the differences in pKa values are not reflected in the chemical shifts of methylated lysines. The rarely occurring buried lysine side chains tend to be deprotonated and display unique chemical shifts (Takayama et al. 2008).

We demonstrate that at pH close to the lysine pKa values the differences in lysine protonation states result in a significant improvement in NMR signal dispersion for methylated lysines. We propose that this effect is general because we could successfully reproduce it on four different proteins including Ca^{2+} -bound Calmodulin (CaM), Lysozyme, Ca^{2+} -bound Troponin C, and Glutathione S-Transferase (GST). In all cases, at pH ~ 7.0 significant NMR signal overlap for lysine methyl groups is observed. This situation is successfully remedied by increasing the pH to 10.0 where the ^1H - ^{13}C HSQC spectra of methyl groups on lysines are well resolved. Thus, the problem of NMR spectral overlap in reductively methylated proteins may be resolved by simply adjusting the pH of the buffering solution. This observation may aid in making reductive methylation more applicable for NMR spectroscopy of proteins that are difficult to express in the isotope enriched form and that are too large to study by conventional NMR. In addition, we show that increased NMR signal dispersion of methyl groups on lysines at pH 10.0 allows to make complete resonance assignments in CaM and to structurally characterize binding of a CaM antagonist trifluoperazine (TFP) to CaM.

Materials and Methods

The proteins used in the study were CaM, Lysozyme, Troponin C and GST. Human CaM was expressed and purified using modifications of previously described protocols (Gopalakrishna & Anderson 1982). Human CaM was cloned into the pET42a expression-vector (Novagen),

using the NdeI and XhoI restriction sites. The protein was expressed under standard conditions in LB media using *E. coli* BL21-A1 cells (Invitrogen). The cells were harvested by centrifugation at 6,000 rpm at 4 °C. The pellet was resuspended in 10 mM Tris/HCl pH 7.6, 5 mM CaCl₂ and 10 mM β-mercaptoethanol and incubated at 65 °C for 30 min. The suspension was centrifuged for 30 min at 18,000 rpm in an SS34 rotor at 4 °C, and the protein was purified using a Phenyl-Sepharose® CL-4B column (Sigma). The column was washed three times with resuspension buffer and the protein was eluted using 10 mM Tris/HCl pH 7.6, 10 mM EDTA and 10 mM β-mercaptoethanol. The protein was dialyzed into 10 mM HEPES pH 7.6, 10 mM CaCl₂ and 10 mM β-mercaptoethanol. Hen egg-white lysozyme (Sigma) was dissolved in 10 mM HEPES pH 7.6 and 10 mM CaCl₂ to a final concentration of 1 mM. Recombinant human cardiac troponin C was expressed and purified according to the method described previously (Kobayashi & Solaro 2006). Purified GST was kindly provided by Dr. Lavie. Troponin C was dialyzed into 10 mM HEPES pH 7.6, 50 mM MgCl₂, 50 mM CaCl₂ and 1 mM β-mercaptoethanol. GST was dialyzed into 10 mM HEPES pH 7.6 and 10 mM CaCl₂.

Reductive methylation of the various proteins used in the study was performed as described (Means & Feeney 1968). Briefly, 20 μL of 1 M borane–ammonia complex (NH₃.BH₃) (Sigma) and 40 μL of 1 M ¹³C formaldehyde (Cambridge Isotope Laboratories, Inc) were added to 1 ml of protein and the reaction was incubated for 2 h with stirring at 4 °C. The addition of borane–ammonia complex and ¹³C formaldehyde was repeated, and the mixture incubated for another 2 h. After the addition of another 10 μL of 1M borane–ammonia complex, the reaction mixture was incubated at 4 °C with stirring overnight. The reaction was stopped by adding glycine to 200 mM, and undesired reaction products as well as excess reagents were removed by dialysis against 10 mM Tris/HCl pH 7.6 and 10 mM CaCl₂ in the case of lysozyme and GST. 1 mM β-mercaptoethanol was also added to the buffer in the case of CaM. Methylated Troponin C was dialyzed against 10 mM Tris/HCl pH 7.6, 50 mM MgCl₂, 50 mM CaCl₂ and 1 mM β-mercaptoethanol.

For NMR experiments, methylated CaM was dialyzed into 40 mM Tris/HCl, 10 mM CaCl₂ and 1 mM β-mercaptoethanol. For comparing spectra at different pH values the buffer pH was adjusted to 7.0, 8.0, 9.0 and 10.0. Methylated Lysozyme and methylated GST were dialyzed into 40 mM Tris/HCl and 10 mM CaCl₂. Methylated Troponin C was dialyzed into 40 mM Tris/HCl, 50 mM MgCl₂, 50 mM CaCl₂ and 1 mM β-mercaptoethanol. For methylated Lysozyme, methylated Troponin C and methylated GST the buffer pH was adjusted to 7.6 and 10.0. The concentrations of the proteins used in the NMR experiments were 150 μM, 250 μM, 200 μM and 90 μM for CaM, Lysozyme, Troponin C and GST, respectively.

The assignments of the ¹³C methyl signals were obtained by point-mutagenesis of individual lysine residues to alanines in CaM. The T26C mutant version of CaM was used in the assignments to provide a site for addition of a paramagnetic probe that will be used in future experiments. Each lysine mutant of CaM T26C was expressed, purified and methylated as described earlier (Gopalakrishna & Anderson 1982). The spectra of the methylated CaM T26C lysine mutants were compared with the spectrum of methylated CaM T26C at pH 10.0 so that each of the nine signals could be assigned to each of the 8 lysine residues and the N-terminus. The effect of binding of TFP to wild-type CaM was studied at pH 7.6 and pH 10.0 by adding 2 molar equivalents of the drug to the protein.

All ¹H–¹³C-edited HSQC spectra were collected at 25 °C on a 600 MHz Bruker spectrometer with a cryogenic probe at 8 scans and 128 indirect points except for methylated GST acquired at 16 scans. The data were processed and analyzed using NMRPipe software (Delaglio et al. 1995).

Results and Discussion

One of the limitations of the use of reductive methylation of lysine residues in biomolecular NMR is poor signal dispersion (Abraham et al. 2008). This phenomenon may be explained by the fact that lysines are commonly surface exposed and therefore are influenced by the invariable environment of the solvent. However, the pKa values of lysines are known to vary significantly in proteins reflecting the effects of the unique amino acid environment surrounding the lysine residues (Zhang & Vogel 1993). Therefore, we hypothesized that by adjusting the pH of the buffering solution it would be possible to unmask the differences between lysine pKa values. We expected that at pH close to lysine pKa different levels of lysine protonation would be reflected in the differences in chemical shifts for the methyl groups introduced by reductive methylation. The proposed approach is illustrated in Figure 1. At pH around 7.0 most lysine side chains are expected to be protonated. Under these conditions we expect significant chemical shift overlap. However, at pH 10.0, which is close to the lysine side chain pKa (Andre et al. 2007), a distribution of differentially protonated states may be observed. Under these conditions differences in pKa values may be unmasked and observed as differences in chemical shifts of lysine methyl groups.

To test the hypothesis that pH adjustment may improve methyl signal dispersion in reductively methylated protein samples we prepared Ca^{2+} -CaM modified with ^{13}C formaldehyde. The degree of incorporation of ^{13}C methyl groups into CaM was tested by MALDI-TOF mass-spectrometry (Abraham et al. 2008), which indicated the presence of eight di-methylated lysines and a di-methylated N-terminus. The two methyl groups on di-methylated lysines possess degenerate chemical shifts, which contributes to the increased sensitivity of NMR signal detection in reductively methylated proteins. The results of pH titration followed by NMR ^1H - ^{13}C HSQC spectra of methyl groups are presented in Figure 2. The results show a pH dependent improvement in NMR signal dispersion. The signal dispersion in the ^1H dimension at pH 7 is 0.4 ppm. In the ^{13}C dimension the signal dispersion is 1.5 ppm (Figure 2a). Only 5 out of expected 8 signals are observed due to a severe signal overlap. As expected, an increase in pH to 8.0 improves signal dispersion to 0.5 ppm and 2 ppm in ^1H and ^{13}C dimensions respectively (Figure 2b). Although the number of resonances does not change at pH 8.0, significant upfield chemical shift perturbations are observed in 4 out of 5 signals. The upfield chemical shift changes in methyl ^1H signals of modified lysines at higher pH are consistent with previous observations (Zhang & Vogel 1993), and reflect the effects of different levels of lysine protonation. A further improvement in chemical shift dispersion in both ^1H and ^{13}C dimensions is observed at pH 9.0 (Figure 2c). The chemical shift dispersion increases to 0.6 ppm in the ^1H dimension and to 3 ppm in the ^{13}C dimension. At pH 9.0, the number of observed signals has increased to 6 out of the 9 expected. The final titration point at pH 10.0 indicates the best condition for NMR signal detection of methyl groups on lysine residues in reductively methylated Ca^{2+} -CaM (Figure 2d). Although the ^1H signal dispersion (0.5 ppm) does not improve, the dispersion in the ^{13}C dimension increases to 4 ppm at pH 10.0. All of the 9 expected signals are now resolved in the ^1H - ^{13}C HSQC spectrum. Therefore, pH 10.0, which is close to the previously reported pKa value for methylated lysine side chains (Zhang & Vogel 1993) represents the optimum condition for NMR detection of methyl groups on reductively methylated CaM. We postulate that at pH 10.0 even small differences in the lysine side chain pKa values produce significantly different levels of protonation of ϵ -amino group of the methylated lysines and this may be detected as differences in chemical shifts of ^{13}C methyl groups in reductively methylated CaM.

Next, we wished to investigate the generality of our observation that pH close to the pKa of lysine side-chains is optimal for NMR resolution of signals belonging to ^{13}C methyl groups in reductively methylated proteins. For this purpose, we have compared the number of signals observed in reductively methylated Lysozyme, Ca^{2+} -bound Troponin C, and GST in ^1H - ^{13}C

HSQC spectra at pH 7.6 and at pH 10.0. The results of this comparison are shown in Figure 3. In all cases we observed an improvement in the number of resolved signals at pH 10.0. In the spectrum of reductively methylated Lysozyme at pH 7.6 only 6 out of 7 expected signals are observed (Figure 3a). Increasing pH to 10.0 allows observation of all of the expected 7 signals in a ^1H - ^{13}C HSQC spectrum (Figure 3b). Similarly, in the spectrum of reductively methylated Ca^{2+} -bound Troponin C at pH 7.6, only 7 out of 11 expected signals are observed (Figure 3c), while at pH 10.0 all 11 signals could be resolved (Figure 3d). The number of expected methyl signals in reductively methylated GST was determined to be 22 by measuring the mass difference between methylated and unmodified GST by MALDI-TOF mass-spectrometry (Figure 1S). At pH 7.6 only 10 out of 22 expected signals were observed in the spectrum of methylated GST (Figure 3e). However, raising the pH to 10.0 allowed observation of 19 signals out of the 22 expected (Figure 3f). Thus, only 3 signals remained unresolved in methylated GST at pH 10.0 as opposed to 12 unresolved signals at pH 7.6.

Based on the results obtained on four different proteins we propose that raising the pH of the buffering solution to the range of pKa values for lysine side chains improves the resolution in NMR spectra of lysine methyl groups in reductively methylated proteins. This strategy overcomes the signal overlap limitation for the use of reductive methylation of proteins in NMR. One potential drawback of this approach is the chemical exchange between the protonated and nonprotonated states that is favorable at pH 10.0. This phenomenon may be responsible for NMR signal line-broadening and decrease in signal intensity. However, in none of the four proteins studied here we observed severe line-broadening and reduction in signal intensity to an extent that would negate the benefits of significantly improved signal resolution. Another potential downside of the proposed approach is the use of non-physiological pH for protein studies. As electrostatic interactions are important for protein folding and intermolecular interactions, alkaline pH may influence protein structure and macromolecular association (Fujita et al. 1998). However, several studies that allowed structural comparison of proteins at different pH indicate that pH 10.0 is not likely to significantly perturb protein structure (Dixon et al. 1991; Harries et al. 2004; Venugopal et al. 1992). The results presented here also do not indicate that protein unfolding at pH 10.0 is a common phenomenon. In all cases we observed increased spectral resolution indicating that methyl groups on lysine residues experience unique electronic environments which is characteristic of a folded protein. If protein unfolding were to occur we would have expected less signal dispersion. However, as a precaution when pH 10.0 is used to enhance NMR signal resolution of methyl groups in reductively methylated proteins it is recommended to use appropriate controls to ensure that the protein fold and activity are retained.

Better resolution of methyl lysine signals may permit NMR signal assignments and may allow for better assessment of structural features of reductively methylated proteins. To demonstrate the utility of better signal dispersion of methyl signals on lysines we first assigned the ^{13}C methyl signals in the ^1H - ^{13}C HSQC spectrum. We have chosen a point mutagenesis approach to assign the methyl resonances. For this purpose eight lysine to alanine mutants were created in CaM T26C using PCR mutagenesis. The proteins were expressed, purified, and reductively methylated. The ^1H - ^{13}C HSQC spectrum for each lysine mutant was acquired at 600 MHz at pH 10.0 and compared to that of CaM T26C. Since the alanine residues can not be reductively methylated, the specific signals representing mutated lysines were missing in the spectra. An example of this assignment strategy is shown in Figure 4a, where the K77A mutant displays the absence of methyl signal on K77. In addition to the missing K77 methyl signal, some small chemical shift changes associated with the K77A mutation are also observed in the spectrum. The disappearance of only one signal in the spectrum of reductively methylated CaM T26C with a lysine mutation means that the chemical shifts of dimethylated lysines (Rayment 1997) are still degenerate. This implies that the improvement in methyl chemical shift dispersion in reductively methylated proteins is not due to the loss of chemical shift degeneracy

in dimethylated lysines at pH 10.0. All lysine methyl signals in CaM were assigned using the point mutagenesis approach and the assignments are shown in Figure 4b. Complete assignments of ^{13}C methyl signals on lysines of CaM would not be possible at pH 7.0 because of significant spectral overlap observed under these conditions (Figure 2a).

Next, we wished to demonstrate that the increased NMR signal resolution in reductively methylated proteins may lead to more precise structural analysis. To achieve this goal we studied binding of a known CaM antagonist TFP to reductively methylated CaM. TFP was chosen because its binding to CaM has previously been characterized by x-ray crystallography (Harmat et al. 2000). Thus, we had an opportunity to validate the technique of reductive methylation for structural characterization of protein-small molecule interaction by NMR and to evaluate the importance of increased spectral resolution in reductively methylated protein samples for enhanced precision of determination of molecular interfaces. Chemical shift changes for ^{13}C methyl groups on lysines in CaM caused by addition of 2 molar equivalents of TFP were monitored by ^1H - ^{13}C HSQC experiments at pH 7.6 and pH 10.0. The chosen amount of the antagonist was necessary to study binding of two molecules of TFP to CaM as previously described (Harmat et al. 2000).

The results of TFP binding experiments are shown in Figure 5. Significant chemical shift changes in K13, K75, and K94 were readily observable at pH 7.6 (Figure 5a). Additional chemical shift perturbations caused by TFP binding may be present as judged by the change in the shape of the signal representing overlapping resonances for K21, K30, K77, K115 and K148. However, these chemical shift perturbations are very hard to measure and assign at pH 7.6. At pH 10.0 additional changes in chemical shifts representing K21 and K30 could be observed (Figure 5b). A reasonable explanation of this observation is that due to spectral overlap in resonances belonging to methyl groups on K21, K30, K77, K115 and K148 identification of K21 and K30 chemical shift perturbations was impossible at pH 7.6. Thus, improved spectral resolution obtainable at pH 10.0 is needed for more complete structural assessment of the effects of ligand binding.

To address the question of whether the observed chemical shift perturbations in methyl groups on lysines of CaM represent structural rearrangements caused by TFP binding we mapped the statistically significant chemical shift changes (>0.03 ppm in ^1H) at pH 10.0 onto the crystal structure of CaM bound to two molecules of TFP (Harmat et al. 2000). The results are shown in Figure 6. As could be observed from the structure of CaM-TFP complex K75, K94, and K21 are in the vicinity of the TFP binding site on CaM. Chemical shift perturbations in K13 and K30 may reflect bending in CaM caused by TFP binding to the hydrophobic pockets of the N- and C-terminal domains. The structural bending of CaM brings together the N- and C-terminal domains resulting in a more compact structure. Thus, the observed chemical shift perturbations in lysine methyl signals in CaM caused by TFP binding may be readily justified based on the crystal structure of the CaM-TFP complex.

Conclusion

In conclusion, we have demonstrated that recording NMR spectra at pH close to the pKa of lysine side chains improves the spectral resolution of lysine ^{13}C methyl groups in reductively methylated proteins. The pH effect on chemical shifts of methyl groups is rationalized by the differences in protonation levels of individual lysine side chains. The benefit of higher pH for NMR spectral resolution of methyl groups in reductively methylated proteins has been successfully reproduced in four different proteins implying the general nature of this approach. This is significant, since the proposed method overcomes one of the major limitations of the use of reductive methylation in NMR and may allow a wider exploration of the advantages offered by reductive methylation in NMR. We also show that improved spectral resolution in

methyl groups of lysines allows accurate resonance assignments and better assessment of structural perturbations in a protein caused by small molecule binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CaM, Calmodulin; HSQC, Heteronuclear Single Quantum Coherence; GST, Glutathione S-Transferase; TFP, trifluoperazine..

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Figure 1.

Schematic showing the di-methylated lysine side chain at two different pH values. When buffer pH is close to 7.0 the ε-amino group is almost always protonated. By changing the buffer pH to around 10.0, which is close to the pK_a value of methylated lysine side chains, the ε-amino group exchanges the proton with solution. Since each lysine side chain has a unique pK_a value depending on its environment, the level of protonation at pH ~ 10.0 would differentially affect the chemical shift of each lysine in a protein.

Figure 2.

^1H - ^{13}C HSQC spectra of reductively methylated Ca^{2+} -CaM comparing signal dispersion at various pH values. At pH 7.0 only 5 out of the 9 expected signals are observed (a). At pH 8.0 the signal dispersion increases in both the ^1H and ^{13}C dimensions though the number of dispersed signals remains the same (b). At pH 9.0 the number of dispersed signals increases to 6 (c). At pH 10 which is close to the pKa value of methylated lysine side-chains, all of the 9 expected signals are well dispersed (d). The spectra were acquired on the 600 MHz Bruker Spectrometer with a cryogenic probe at 25 °C.

Figure 3.

Comparison of ^1H - ^{13}C HSQC spectra of three different proteins at pH 7.6 and pH 10.0. In methylated Lysozyme 6 out of the expected 7 signals are observed at pH 7.6 (a), while at pH 10.0 all of the expected 7 signals are well dispersed (b). In methylated Ca^{2+} -bound Troponin C, only 7 out of the expected 11 signals are observed at pH 7.6 (c), while at pH 10.0 all of the expected 11 signals are well dispersed (d). In methylated GST only 10 out of the expected 22 signals are observed at pH 7.6 (e), while 19 out of the expected 22 signals are observed at pH 10.0 (f).

Figure 4.

(a) An example of lysine point mutation of methylated CaM. ^1H - ^{13}C HSQC spectrum of CaM T26C K77A double mutant shows one missing methyl resonance, indicated by '*'. The missing signal is assigned to K77 by comparison with the spectrum of reductively methylated CaM T26C. (b) The ^1H - ^{13}C HSQC spectrum of methylated CaM T26C shows all 9 methyl signals that were assigned using the individual lysine point mutants of CaM T26C.

Figure 5.

(a) Overlay of ^1H - ^{13}C HSQC spectra of methylated wild-type CaM (blue) and methylated wild-type CaM bound to 2 molar equivalents of TFP (red) at pH 7.6. Methyl resonances belonging K21, K30, K77, K115 and K148 are overlapping at pH 7.6 and cannot be used for structural assessment. (b) Overlay of ^1H - ^{13}C HSQC spectra of methylated wild-type CaM (blue) and methylated wild-type CaM bound to 2 molar equivalents of TFP (red) at pH 10.0. Since all of the 9 methyl resonances are well resolved, changes in specific chemical shifts namely K13, K21, K30, K75 and K94 can be identified.

Figure 6.

Statistically significant chemical shift changes (>0.03 ppm in ^1H) in the ^1H - ^{13}C HSQC spectrum of methylated wild-type CaM bound to TFP at pH 10.0 were mapped onto the crystal structure of CaM-TFP complex with 2 molecules of TFP (Harmat et al. 2000). The CaM molecule is shown as a grey ribbon. The two molecules of TFP are colored green. The lysine side chains that show significant perturbations in the ^1H - ^{13}C HSQC spectrum of methylated CaM-TFP are colored red. The assignments of these lysines are indicated in the figure.