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ARTICLE *in* JOURNAL OF PHYSICAL CHEMISTRY LETTERS · SEPTEMBER 2011

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J Phys Chem Lett. 2011 September 1; 2(17): 2158–2162. doi:10.1021/jz200980g.

Azido Homoalanine is a Useful Infrared Probe for Monitoring Local Electrostatistics and Sidechain Solvation in Proteins

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Abstract

The use of IR probes to monitor protein structure, deduce local electric field, and investigate the mechanism of enzyme catalysis and protein folding has attracted increasing attention. Here, the azidohomoalanine (Aha) is considered as a useful IR probe. The intricate details of the distinct effects of backbone peptide bonds and H-bonded water molecules on the azido stretch mode of the IR probe Aha were revealed by carrying out QM/MM MD simulations of two variants of the protein NTL9, NTL9-Met1Aha and NTL9-Ile4Aha and comparing the resulting simulated IR spectra with experiments.

Keywords

IR probe; azidohomoalanine; QM/MM MD simulation; mutated proteins; protein structure; protein dynamics

IR probes provide information on protein conformation, the structure of amyloid fibers, protein dynamics, DNA structures, and the role of electric fields in enzyme catalytic active sites. [1–15] However, most probes suffer from low sensitivity due to their small oscillator strengths[4] and they can be difficult to introduce into proteins in a site-specific manner. These factors have limited their extensive use for monitoring dynamical changes of protein structures. Recently, nitrile-, thiocyanato-, and azido-derivatized amino-acids have been found to be useful IR probes because their IR absorbance is in a comparatively transparent region of an aqueous protein IR spectrum and because they can be incorporated into proteins in a site-specific manner.[4,8,16–20] Furthermore, the CN and N_3 stretch mode frequencies and their IR band shapes are quite sensitive to the environment and to H-bonding interactions.[21–22]

We have shown that the azido group in azidoalanine or C_γ-substituted azidoproline peptides actively participates in intramolecular electrostatic interactions with backbone peptides, with stereoelec-tronic effects on the intrinsic peptide conformational propensities.[23] In comparison, Azidohomoalanine (Aha), with an additional methylene group at the C_β-position, is likely to be a better IR probe because the N_3 oscillator is more isolated from the

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Supporting Information. Computational details; Figures S1–S4. This material is available free of charge via the Internet at http://pubs.acs.org.

polar peptide bonds due to the increased distance of the azido group from the polypeptide backbone. Taskent-Sezgin et al. demonstrated that this probe is easily incorporated into proteins using Met auxotrophic strains. They used the 56-residue N-terminal domain of the ribosomal protein L9 (NTL9), which has been extensively studied as a model system for protein folding and stability, as a test case.[24–27] They obtained the azido stretch IR spectra of two mutants, NTL9-Met1Aha and NTL9-Ile4Aha and showed that the mutations do not significantly perturb the protein. This work qualitatively demonstrated that the local electrostatic environment of the azido group in the NTL9-Met1Aha mutant is significantly different from that in the NTL9-Ile4Aha.[17] However, a quantitative analysis of the factors that influence the band position and line width was not possible. This information is essential if Aha is to be used as a site specific probe. It was believed that the azido group in the Met1 mutant is close to the hydrophobic core, whereas the azido group in the Ile4 mutant is partially exposed to solvent water due to fraying of the C-terminal helix in the protein. However, the roles of local electrostatics and water molecules H-bonded to the azido IR probe could not be elucidated.

Knowledge of the relative importance of these two effects is essential for the quantitative interpretation of azido IR spectra and is required if Aha is to be used as a site specific probe. To address these issues, we carried out quantum mechanical/molecular mechanical molecular dynamics (QM/MM MD) simulations (see Supporting Information for details) of NTL9-Met1Aha and NTL9-Ile4Aha, determined statistical distributions of H-bonded water molecules, obtained azido stretch mode frequency trajectories, and calculated the frequency-frequency correlation function and the corresponding azido stretch IR spectra. Aha was treated quantum mechanically using the AM1 approximation and the remaining peptide groups and solvent water (TIP3P) were taken into account classically using the force field implemented in the AMBER 9 program.[28] This study provides a protocol for the analysis and interpretation of the spectra of azido labeled IR proteins. The resulting confluence of simulated spectra with experimental observations is found to be good.

The structures of Aha, Met, and Ile are shown in Figure 1(a). Aha is approximately isosteric for Met but not for Ile, even though the volume of Aha is reasonably similar to that of Ile. The ribbon diagram of NTL9 in Figure 1(b) highlights the location of the two sites, Met1 and Ile4, which were replaced with an Aha. The resulting proteins are designated NTL9-Met1Aha and NTL9-Ile4Aha, respectively. QM/MM MD trajectories were used to examine the immediate environments of the azido groups in NTL9-Met1Aha and Ile4Aha and representative zoomed-in structures are shown in Figures 1(c) and (d). The azido group in Met1Aha appears to point into the non-polar pocket, whereas that in Ile4Aha is largely exposed to solvent water. However, there are still one or two water molecules forming hydrogen-bonding interactions with the inner N-atom of the azido group of Aha in Met1Aha. In addition, the middle N-atom in the azido group in NTL9-Met1Aha, which has a positive atomic partial charge, experiences electronic interactions with the carbonyl oxygen atom of Met1. In contrast, the azido group in Ile4Aha exclusively forms hydrogen-bonds with surrounding water molecules. In order to examine the water distribution around the azido IR probes in the two mutants, we calculated the pair correlation functions of the water H-atoms around the terminal and inner N-atoms of the azido groups. The results are displayed in Figures 2(a) and (b). Surprisingly, the water distribution around the terminal N-atom in Met1Aha is little different from that in Ile4Aha, despite the fact that the azido group in Met1Aha was believed to be inside a non-polar pocket. Interestingly, the probability of finding hydrogen-bonded water molecules with the inner N-atom of the azido group in Met1Aha is significantly larger than that in Ile4Aha. The radial water distributions are, however, not enough to shed light onto the origin of the sol-vatochromic frequency shifts of the azido groups in the mutants.

We recently showed that the azido stretch mode frequency is highly sensitive to detailed H-bond angle θ defined as $\angle$N–N…H(H_2O).[22] More specifically, for the terminal N-atom of an azido group, an angle θ larger (smaller) than ~130° causes a frequency blue (red)-shift of the stretch mode. On the other hand, H-bonding to the inner N-atom induces a blue-shift. Therefore, the H-bond angle distributions as well as the number distribution of the H-bonded water molecules around the two N-atoms in each azido group are critical factors determining the hydration-induced frequency shift of the azido IR probe. Figure 2(c) depicts the H-bond angle distributions around the azido terminal N-atoms in Met1Aha and Ile4Aha. Notice that the corresponding peak positions are found to be 130° and 150°, respectively. This indicates that the azido group inside the non-polar pocket of Met1Aha is not flexible enough to allow the azido group's terminal N-atom to form an optimum H-bond interaction with a water molecule. On the other hand, for the azido group in Ile4Aha, the terminal N-atom makes a strong and optimum H-bond with water. In contrast, there is no notable difference in the distributions of the H-bond angles at the inner N-atomic site, except that the average number of water molecules H-bonded to the inner N-atom in Met1Aha is slightly larger than that in Ile4Aha.

The statistical analyses of the QM/MM MD trajectories provided valuable information about the local hydration environment of the azido IR probes, but such information does not directly allow us to obtain spectroscopic data that can be compared with experiments. We therefore used a theoretical model based on the distributed multipole analyses of vibrational solvatochromic frequency shifts of IR probes.[22,29–31] An essential concept in this approach is that the distributed interaction sites around the vibrational chromophore (see Supporting Information for a detailed description on the theoretical model) collectively act like an antenna detecting the local electrostatic potential that is produced by surrounding medium including solvent and other groups in the protein.[29] Using azidomethane as a model system, we showed that such a distributed interaction site model worked well for quantitatively determining the azido stretch mode frequency shift upon solvation.[22] This model was further verified by conducting infrared spectroscopic investigations of (4R)- and (4S)-azido-proline peptides in solutions, where an intramolecular electrostatic interaction between the C_γ-substituted azido group and a backbone peptide bond leads to a blue shift of the azido stretch mode (unpublished results). These two examples suggest that we need to take into account two important contributions simultaneously, namely interactions with solvent water as well as interactions with neighboring polar carbonyl groups in the peptide. To calculate the azido frequency for each individual snapshot configuration sampled from the QM/MM MD trajectories,[22] all we need are (i) the solvatochromic frequency parameters previously determined for the azido stretch mode, (ii) water and peptide coordinates extracted from the trajectory files, and (iii) atomic partial charges of water and proteins (here we carried out a density function theory calculation, with the B3LYP/6-311++G(3df,2pd) basis set, of N-methylacetamide to determine the atomic partial charges of the protein peptide bond). It should be mentioned that the side-chains of the amino-acids, Val3, Val21, Ala26, and Phe31, in the non-polar pocket of the Met1Aha were found to contribute negligibly to the azido stretch mode frequency shift so that they were ignored in the calculations. There is a polar amino-acid, Asp23, close to the pocket, but it is fully solvated by water and its side-chain is far from (> 5.0Å) the azido group.

Since the azido frequency shift is given as a sum of contributions from backbone peptides and water molecules, we could separately examine each distribution of frequency shifts compared to the gas-phase value of the azido stretch frequency ν_0. Figure 3(a) for Met1Aha shows that the H-bonded water molecules lead to an azido frequency red-shift of 6.9 cm^{-1}, whereas the Coulomb interaction between the Met1 peptide carbonyl oxygen atom and the azido group's middle N-atom causes a strong blue-shift of about 12.5 cm^{-1}. Interestingly, the two contributions largely cancel out and result in a small blue-shift (5.7 cm^{-1}) of the

azido stretch mode in Met1Aha relative to the gas-phase value. This illustrates the dangers of interpreting spectral shifts in proteins on the basis of empirical correlations observed using simple model compounds in neat solvents. In contrast, in the case of Ile4Aha, the peptide contribution to the azido frequency shift is much smaller than the water contribution (Figure 3(b)). The optimum H-bonded water molecules at the terminal N-atom in Ile4Aha causes a strong blue-shift by 8.4 cm^{-1} and the total blue-shift is estimated to be 9.7 cm^{-1}. This means that the azido frequency difference between Met1Aha and Ile4Aha is about 4 cm^{-1}. Although this result is smaller than the experimental result (11 cm^{-1}),[17] the general trend is well-reproduced. The key observation is that the frequency shift of the Met1Aha mutant is caused by a delicate balance between the contributions from the water and from the nearby backbone peptide groups. This point will remain valid regardless of computational method used. The complicated nature of the solvatochromism of Aha makes this IR probe highly specific and sensitive to small differences in the local electrostatic environment in proteins.

Despite the fact that the azido frequency distributions were obtained from the simulated trajectories, they do not correspond to the azido stretch IR spectra due to spectral diffusion and motional narrowing effects. Thus, we next calculated the fluctuating frequency-frequency correlation function (FFCF), defined as $M(t) = \langle \delta\Omega(t)\delta\Omega(0)\rangle$ where $\delta\Omega(t) = 2\pi c\{\delta\tilde{\nu}(t) - \langle\delta\tilde{\nu}\rangle\}$. The $M(t)$'s of Met1Aha and Ile4Aha show a bimodal decay (Figures 4(a) and (b)). Spectral densities obtained from the $M(t)$'s (see Figure S4) reveal that the fast (<100 fs) component is produced by hindered translational and rotational motions of the H-bonded water molecules. The slow component is attributed to the overall azido group rotation, the H-bond making and breaking dynamics of water, and slow side-chain motions. As expected, the FFCF of Ile4Aha is dictated by the water contributions for the entire timescale. In stark contrast, the contributions from peptide and water to the FFCF of Met1Aha are both fairly large up to picosecond timescales. Notably, the peptide contribution to the FFCF of Met1Aha does not decay to zero even at long times (~2 ps), which indicates that the intramolecular interaction dynamics between the Met1 carbonyl group and the azido group's middle N-atom is a slowly varying process. The analysis makes it clear that the local solvent and neighboring peptide interactions with the azido group result in spectroscopic changes of the azido stretch modes and lead to distinctively different IR spectra.

Once the FFCF is obtained, the corresponding IR absorption spectrum can be calculated by performing the following Fourier transformation,[32]

$$I(\omega) \propto \int_{-\infty}^{\infty} dt\, e^{i(\omega-\bar{\omega})t} \exp[-g(t) - t/2T_1]. \quad (1)$$

Here the line-broadening function $g(t)$ is determined by the Fourier spectrum of the FFCF (see SI for detailed description), and $\bar{\omega}$ and T_1 are the ensemble average fundamental transition frequency and the lifetime of the vibrational excited state, respectively. The numerically calculated frequencies are multiplied by a scaling factor of 0.9410, which was determined by comparing the DFT-calculated frequency of azido methane and the experimentally measured azido stretch frequency of azidoalanine in water.[22] In Figure 4(c), the simulated azido stretch IR spectra of Met1Aha (blue dashed lines) and Ile4Aha (red dashed lines) are plotted, where the pure dephasing contribution was only taken into consideration. The FWHM (full width at half maximum) of the Met1Aha spectrum is found to be 13 cm^{-1}, which is narrower than that (18 cm^{-1}) of the Ile4Aha spectrum. This indicates that the time-scale of the azido-water interactions in Ile4Aha are faster than in Met1Aha. This is fully consistent with the fact that the azido group in Ile4Aha is exposed to water. Interestingly, even without the additional lifetime broadening contribution, the

FWHM value of the simulated Met1Aha IR spectrum is close to the experimental result, 13 cm^{-1}. However, the FWHM of the Ile4Aha peak is significantly smaller than the experimentally measured line width of 32.4 cm^{-1}. Consequently, it is inferred that the vibrational lifetime of the azido stretch mode in Ile4Aha is shorter than that in Met1Aha. Assuming that the corresponding lifetimes are 3.0 and 0.35 ps for t Met1Aha and Ile4Aha, we re-calculated the IR spectra (solid lines) and plot them in Figures 4(c). Although the peak frequencies of simulated IR spectra deviate from the experimental results, the general trends are all reproduced by the present calculations.

In summary, our calculations of the vibrational solvatochromic frequency shifts of the azido stretch modes in Aha-containing proteins in combination with QM/MM MD simulations provided intricate details of the local electrostatic environment around the azido IR probe in proteins. The approach allows us to deconvolve the effects of interactions with the peptide backbone and other protein groups from the effects of interactions with the solvent. This provides an explanation for the observation that the azido group of Met1Aha experiences a small blue shift despite being located in a hydrophobic pocket in the Met1Aha protein. In this case, interactions with neighboring peptide groups and nearby water molecules induce a small blue-shift of the azido stretch mode in comparison to the gas phase value. In contrast, the azido stretch mode frequency is exclusively determined by hydration in the case of the Ile4Aha. These computational results provide critical insights into the detailed mechanism and the nature of solvatochromic frequency shift of the azido IR probe in azidohomoalanine. We therefore anticipate that this approach will be of use in analyzing spectral shifts of azidohomoalanine in proteins

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NRF (20090078897) and KBSI (T31401) to MC and from the NSF CHE 0806433 and NIH GM078114 to DPR.

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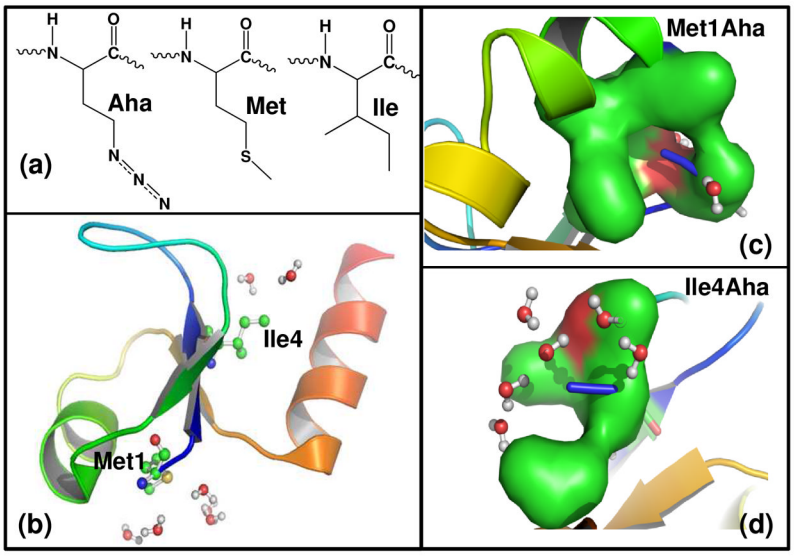

Figure 1.

(a) Molecular structure of azidohomoalanine (Aha), methionine (Met), and isoleucine (Ile). Ribbon diagram of NTL9 (b). The local environment of Met1 and Ile4 are shown. The azido group in NTL9-Met1Aha is in the hydrophobic pocket, though it interacts with a neighboring peptide carbonyl group (shown as red surface inside the pocket) (c). The azido group in NTL9-Ile4Aha points toward solvent water so that it is fully hydrated by surrounding water molecules (d).

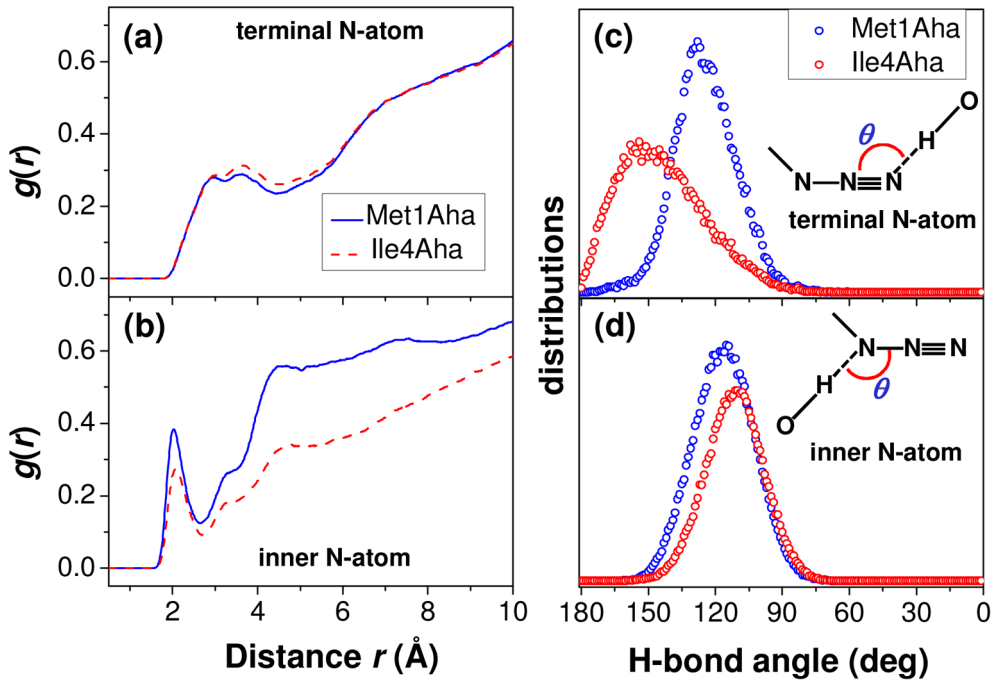

Figure 2.

Pair correlation function, $g(r)$, between the water H-atom and the terminal (a) and inner (b) N-atoms in azido groups. Distributions of H-bond angles (θ), defined as ∠N–N…H, are plotted in (c) and and (d)

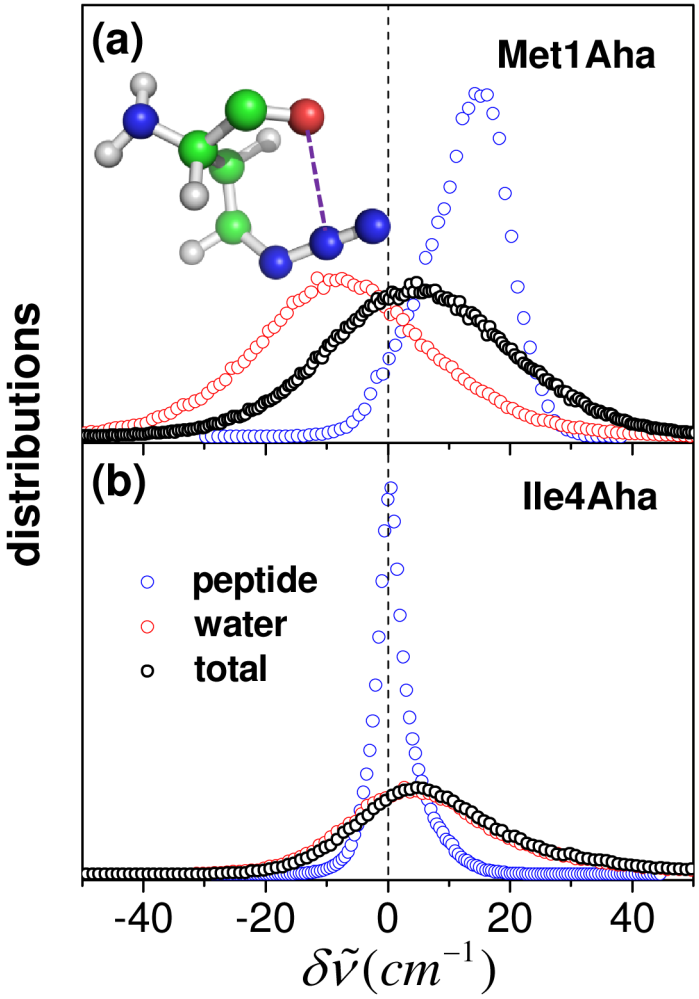

Figure 3.
Frequency shift distributions obtained from the azido frequency trajectories of NTL9-Met1Aha (a) and NTL9-Ile4Aha (b). The contributions from peptides and water are separately plotted as blue and red circles. Total frequency shifts are represented by black circles.

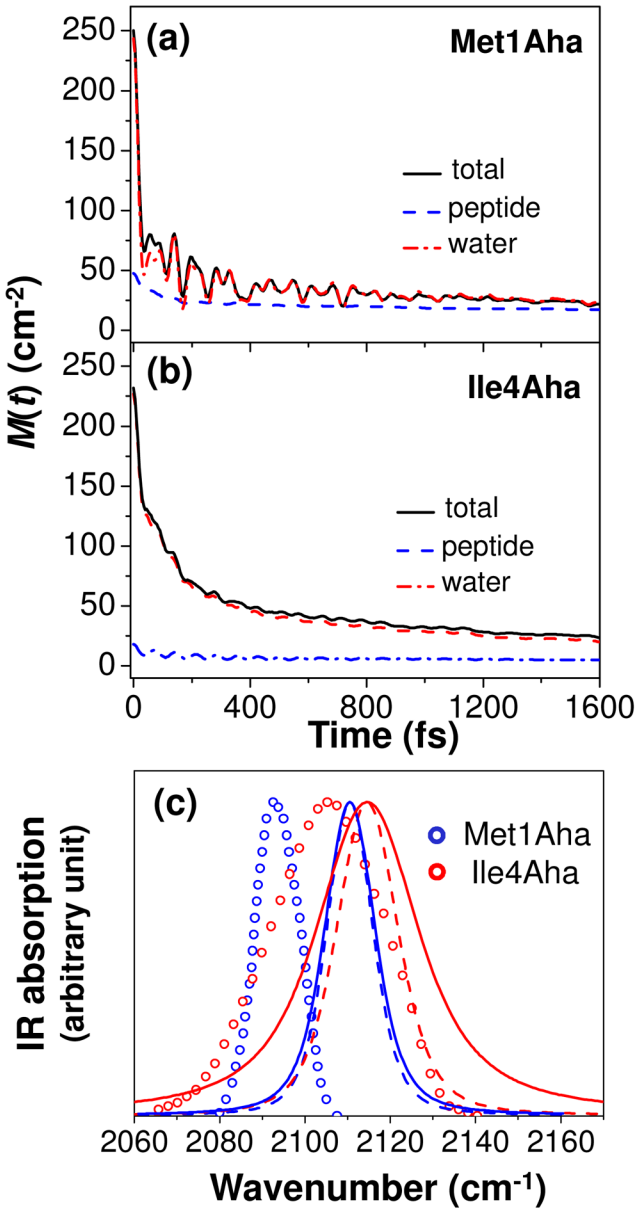

Figure 4.

Frequency-frequency correlation functions (black solid lines) of the azido stretch modes in Met1Aha (a) and Ile4Aha (b). The azido stretch frequency shift is given as the sum of water (red dash-dot) and peptide (blue dashed) contributions so that the FFCF is separated into the corresponding two terms. Here, the cross-correlation function is found to be negligibly small. In Figure (c), the numerically simulated IR spectra with (solid) and without (dashed) lifetime broadening contributions are plotted and compared with experimental results (open circles).