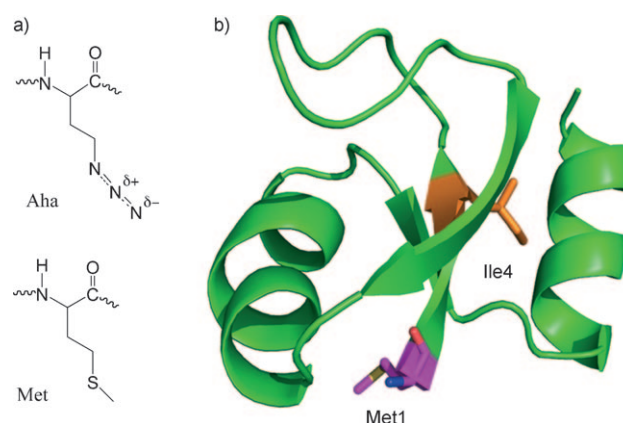


# Azidohomoalanine: A Conformationally Sensitive IR Probe of Protein Folding, Protein Structure, and Electrostatics\*\*

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IR probes are widely used to study protein dynamics,<sup>[1]</sup> protein–ligand interactions, and electric field effects in proteins, but such probes can suffer from poor sensitivity or can be difficult to introduce.<sup>[1a,f,2]</sup> The approach requires the introduction of an IR active reporter group into the protein of interest. Here we show that an azido-bearing nonnatural amino acid, azidohomoalanine (Aha), provides a high-sensitivity probe of protein structure, protein folding, and protein electrostatics. Interest in the azido group was initially driven by its application in bioorthogonal protein labeling and click chemistry.<sup>[3a]</sup> Use of the azido group as an IR probe has a number of attractive features. Its absorbance falls in an otherwise transparent region of the IR spectrum and the frequency is sensitive to the environment.<sup>[4]</sup> Of particular importance, the extinction coefficient of an azido group is approximately twenty-fold larger than that of the commonly employed cyano group.<sup>[4a]</sup> Azidohomoalanine (Aha) can be viewed as an analogue of Met and, thus, one can realize its incorporation using well developed Met auxotrophic strains (Figure 1a).<sup>[3]</sup> The group is also compatible with the conditions of Fmoc solid-phase peptide synthesis.

We used the N-terminal domain of the ribosomal protein L9 (NTL9) as a test case. NTL9 is a 56-residue protein, with a mixed  $\beta$ – $\alpha$  structure that has been studied extensively as a model system for protein folding and stability. It folds cooperatively under a wide range of conditions.<sup>[5]</sup> We targeted Met1 by expression (NTL9-Met1Aha) and Ile4 (NTL9\*-Ile4Aha) by chemical synthesis as sites for incorporation of the Aha substitutions (Figure 1b). NTL9\* refers to a K12A



**Figure 1.** a) Structure of azidohomoalanine (Aha) and methionine (Met). b) A ribbon diagram of NTL9 showing the location of Met1 (pink) and Ile4 (orange). The diagram was generated using the pdb file 2HBA and the program Pymol.

mutant of NTL9 which adopts the same fold, but is more stable than the wild-type. Both of these positions are in the hydrophobic core of the protein (Figure 1b).

Azidohomoalanine was synthesized in four steps starting from homoserine in 17 % overall yield (Supporting Information). Protein expression was performed with the standard methionine auxotrophic *E. coli* strain, B834. Mass spectrometry was used to test the level of incorporation. The experimental and theoretical mass spectra of NTL9-Met1Aha match extremely well and a minimum incorporation of 94 % was detected by MS–MS analysis (Supporting Information). The small amount of wild-type protein is spectroscopically silent in the azido vibration region of the IR spectrum.

Spectroscopic analysis shows that the substitution does not perturb the structure of the protein. The far-UV CD spectrum of the M1Aha mutant is similar to the spectrum of the wild-type indicating that they have similar secondary structure. The 1D-NMR spectrum of the mutant displays the characteristic ring current shifted methyl resonances of NTL9, as well as  $\alpha$  proton chemical shifts downfield of  $\text{H}_2\text{O}$ , indicative of  $\beta$ -sheet structure, and the NOE spectrum shows that the native registry of the  $\beta$ -sheet is maintained (Supporting Information). The potential effects of the substitution on the stability and cooperativity of folding were also examined. Urea denaturation shows a sigmoidal curve, as expected for a cooperatively folded unit. The stability is decreased relative to the wild-type by  $0.81 \text{ kcal mol}^{-1}$ . The  $m$ -value, which is the slope of the  $\Delta G^\circ$  vs [urea] curve, is very

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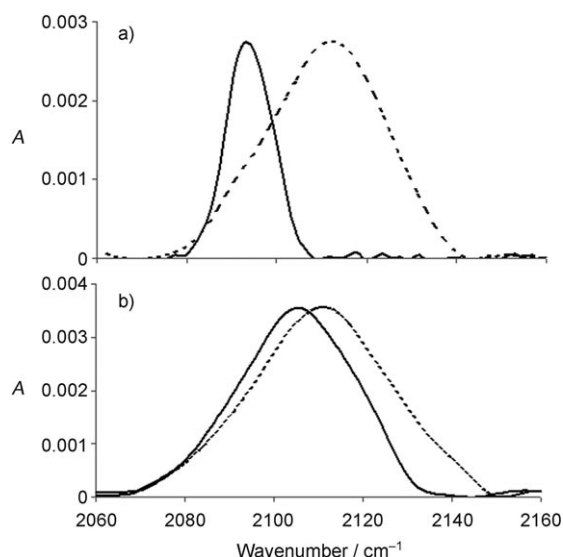
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[\*\*] This work was supported by NSF grants CHE 0806433 to D.P.R., CBET 1080909 to I.C. and NIH grant GM053640 to R.B.D. We thank Dr. Lisa Miller and Megan Bourassa from Brookhaven National Laboratory for assistance in collecting preliminary data, and Wenli Meng for his help with the NMR experiments. We also thank Dr. Antonius Kohler from the Stony Brook University Proteomics Center for help with the mass spectrometry. The proteomics center is supported by NIH/NCRR 1 S10 RR025072-1.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/ange.201003325>.

similar to that of the wild-type.  $m$ -values are widely believed to be related to the change in accessible surface area between the folded and unfolded states and the good agreement between wild-type and mutant provides additional evidence of the structural integrity of the mutant.<sup>[6]</sup> Thermal unfolding is also cooperative and the melting point ( $T_m$ ) is decreased by 5 to 6°C (Supporting Information). Stopped-flow kinetic refolding studies confirm that folding is two-state (Supporting Information).

IR spectra were recorded in the folded state and in the unfolded state induced by temperature. The folded-state spectrum was recorded at pD 8.8 to ensure a single protonation state for the N-terminus since a partially protonated N-terminus might lead to a splitting of the folded-state peak due to electrostatic effects. NTL9 is fully folded at pD 8.8, 20°C (Supporting Information). In the folded state, the azido band is at 2094 cm<sup>-1</sup>. In the unfolded state at 90°C, a single broad band is found at 2113 cm<sup>-1</sup>, indicating exposure of the azido group to water (Figure 2). The significant band shift observed between the folded and unfolded states indicates that the azido vibration can be used as a probe of protein folding and hydrophobic burial of side chain.



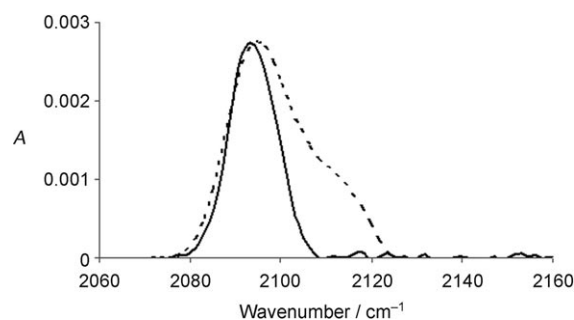
**Figure 2.** FTIR spectra of a) NTL9-Met1Aha and b) NTL9\*-Ile4Aha in the folded state at 20°C (continuous line) and in the unfolded state at 90°C (broken line).

The methodology is not limited to selective incorporation of a Met at the N-terminus since methionyl aminopeptidase has been shown to cleave azidohomoalanine from proteins expressed in *E. coli* depending on the solvent exposure of Met1 and the amino acid following Met1. Methionyl aminopeptidase cleaves N-terminal azidohomoalanine if the next amino acid is small.<sup>[3b]</sup> In NTL9, Met1 is part of the hydrophobic core and is retained when the protein is expressed in normal cell lines.

We demonstrated that the probe can also be incorporated into proteins by solid-phase peptide synthesis. Ile4 in NTL9

was replaced by Aha using Fmoc based methods (Supporting Information). The Ile4Aha variant adopted the same fold as wild-type as judged by CD, 1D, and 2D-NMR spectroscopy. Thermal and denaturant induced unfolding are still cooperative and stopped-flow kinetic studies confirm that folding is two-state (Supporting Information). Substitution of Aha for Ile4 is more destabilizing than for Met1 with a  $\Delta\Delta G^\circ$  of 1.9 kcal mol<sup>-1</sup> (Supporting Information). The larger effect is likely due to the fact that Aha is approximately isosteric for Met but not for Ile. IR spectra of the Ile4Aha mutant were taken in the folded state and in the thermally unfolded state. In the folded state the azido vibration was observed at 2105 cm<sup>-1</sup> and in the unfolded state the vibration blue-shifts to 2112 cm<sup>-1</sup> (Figure 2). The observed frequency for Aha in the folded state of the Ile4Aha mutant at 20°C indicates that azido group is more exposed to solvent than in the Met1Aha mutant. Ile4 packs against the C-terminal helix and this helix is known to fray at its C-terminus in the wild-type. The change in shape and polarity of the substitution may enhance this effect in the mutant and account for the partial solvent exposure. Irrespective of the details, the experiments with Ile4Aha demonstrate that Aha can be incorporated by solid-phase peptide synthesis and reiterate that the IR vibration of the azido group is sensitive to its environment.

Spectra were also recorded of the Met1Aha variant at pD 6.0 to test the sensitivity of the probe to varying nearby charges. NTL9 is fully folded under these conditions but the N-terminus is partially protonated. Figure 3 compares the spectrum recorded at pD 6.0 to that measured at pD 8.8. The pD 6.0 spectrum displays a second partially resolved peak at 2116 cm<sup>-1</sup> which is due to the protonated form (Supporting Information) and a major peak at 2094 cm<sup>-1</sup>. The drastic change observed upon partial protonation of the N-terminus demonstrates the sensitivity of the azido group vibrational mode to changes in electrostatic effects and minor native state structural variations.



**Figure 3.** FTIR spectra of the folded state of NTL9-Met1Aha at pD 6.0 (broken line) and pD 8.8 (continuous line).

We have demonstrated that azidohomoalanine is a sensitive IR probe of protein folding, protein structure, and electrostatic effects. The probe can be easily incorporated into proteins in high yield in a site-specific manner using simple, readily available auxotrophic expression systems as well as by solid-phase peptide synthesis. The probe provides a complementary approach to methods involving orthogonal amino-

acyl tRNA synthetases or approaches that involve the attachment of probes to introduced cysteine residues and should be generally accessible.<sup>[1d,2c]</sup>

Received: June 1, 2010

Published online: September 2, 2010

**Keywords:** azidohomoalanine · electrostatics · infrared spectroscopy · protein folding · protein modifications

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