The Effects of α-Helical Structure and Cyanylated Cysteine on Each Other

Lena Edelstein, Matthew A. Stetz, Heather A. McMahon, and Casey H. Londergan*

Department of Chemistry, Haverford College 370 Lancaster Avenue, Haverford, Pennsylvania 19041-1392

Received: February 16, 2010

β-Thiocyanatoalanine, or cyanylated cysteine, is an artificial amino acid that can be introduced at solvent-exposed cysteine residues in proteins via chemical modification. Its facile post-translational synthesis means that it may find broad use in large protein systems as a probe of site-specific structure and dynamics. The C≡N stretching vibration of this artificial side chain provides an isolated infrared chromophore. To test both the perturbative effect of this side chain on local secondary structure and its sensitivity to structural changes, three variants of a model water-soluble alanine-repeat helix were synthesized containing cyanylated cysteine at different sites. The cyanylated cysteine side chain is shown to destabilize, but not completely disrupt, the helical structure of the folded peptide when substituted for alanine. In addition, the C≡N stretching bandwidth of the artificial side chain is sensitive to the helix−coil structural transition. These model system results indicate that cyanylated cysteine can be placed into protein sequences with a native helical propensity without destroying the helix, and further that the CN probe may be able to report local helix formation events even when it is water-exposed in both the ordered and disordered conformational states. These results indicate that cyanylated cysteine could be a widely useful probe of structure-forming events in proteins with large in vitro structural distributions.

Introduction

Vibrational spectroscopy could be a very useful probe of both structure and dynamics in proteins, but its site-specificity is usually limited by spectral overlap of the normal modes of common biomolecular functional groups. Two common strategies to isolate specific protein sites in vibrational spectra are isotopic labeling, usually of carbonyl groups along the amide backbone[1−5] but sometimes of side chains,[6−9] and the introduction of novel functional groups on selected side chains. A recently popularized functional group is the nitrile (C≡N) moiety, whose stretching frequency appears in an otherwise clear region of the biomolecular infrared spectrum.[10] This group has been introduced using the artificial amino acids *p*-cyanophenylalanine,[11,12] β-cyanoalanine,[11] and 5-cyanotryptophan;[13] it has also been used as a site-specific probe when located on substrate molecules that bind to selected target proteins.[14−16] In each of these cases, the nitrile moiety is usually synthetically incorporated into the peptide or substrate: this synthetic requirement limits the number of protein systems that can be addressed using site-specific vibrational spectroscopy of nitrile groups. An exception is *p*-cyanophenylalanine, which has been successfully incorporated in two cases using nonsense expression.[17,18]

An alternative approach to incorporation of the CN group is chemical modification of naturally occurring side chains. Cyanylation of the phenol oxygen of tyrosine yields the cyanate (OCN) moiety, whose C≡N stretching band is complicated by Fermi resonances.[19] Covalently attached C≡N can also be introduced via post-translational chemical modification of free cysteine thiols to thiocyanate, yielding the artificial amino acid β-thiocyanatoalanine, or cyanylated cysteine.[14,20−23] Covalent modification of side chains can in principle be used to provide site specificity in larger and more diverse protein systems than synthetic incorporation of artificial amino acids. In the case of cyanylated cysteine, one or more solvent-exposed cysteine residues are required: these may be inserted via site-directed mutagenesis if not already present in the sequence of interest. It has been shown that the frequency and line shape of the C≡N stretching band of aliphatic thiocyanate are sensitive to the hydrogen bonding and dynamic properties of the local structural environment.[24] Quantum chemical calculations[25] and model compound studies[24] also indicate that the aliphatic SC≡N moiety does not tend to form strong hydrogen bonds to conventional functional groups in its surroundings, and thus the cyanylated cysteine side chain should have a relatively weak structural influence on the local environment. Here, the sensitivity of this vibrational probe to well-defined structural changes in simple helical peptides is documented, with the goal of establishing the probe vibration's utility in proteins with especially disordered or dynamic structures.

Protein sequences with no predicted structure may make up more than 50% of the human proteome;[26] it has been noted that organisms of increasing complexity contain a greater proportion of unstructured sequences. Proteins whose functional forms contain no clear secondary structure are called intrinsically disordered proteins (IDPs).[27] These proteins frequently display the ability to bind to many biological partners, often accompanied by a gain of structure on binding.[28−30] A subset of characterized IDPs display random coil to helix transitions in subdomains with binding activity.[28,31] These subdomains can display nascent helical character that is enhanced upon exposure to backbone-dehydrating agents like 2,2,2-trifluoroethanol. A useful site-specific probe of such local coil-to-helix transitions should be able to report the structural transition without perturbing it to a large extent. Site-directed spin labeling, which usually involves attaching a paramagnetic probe group via disulfide bond at free cysteine residues,[32] is currently a popular technique for mapping disorder−order transitions, but it has the drawback that the relatively bulky paramagnetic probe group can disrupt binding interfaces and hydrophobic contacts in bound conformations.[33,34] Here we examine the cyanylated cysteine

* Corresponding author. E-mail: clonderg@haverford.edu.

10.1021/jp101447r © 2010 American Chemical Society
Published on Web 03/18/2010

TABLE 1: Amino Acid Sequences of Alanine-Repeat Peptides, Where C* Indicates Cyanylated Cysteine

peptide sequence	abbreviation
Ac-AAAAKAAAAKAAAAKAAAAKAAAAK-NH_2	AAA
Ac-A**C***AAKAAAAKAAAAKAAAAKAAAAK-NH_2	CAA
Ac-AAAAKAAAAKA**C***AAKAAAAKAAAAK-NH_2	ACA
Ac-AAAAKAAAAKAAAAKAAAAKA**C***AAK-NH_2	AAC

vibrational probe in the context of a well-characterized helical peptide that displays a clear helix−coil transition, partly to examine the perturbing effect of the relatively small probe moiety on an otherwise very helical sequence.

Alanine-rich peptides have long been known to prefer an α-helical conformation;[35] alanine has the most native helical propensity of any amino acid in both short peptides and natural proteins.[36−39] Alanine-rich peptides with the sequence $(AAAAX)_n$, where X is a charged or polar amino acid, were developed as water-soluble peptides to model ideal helical structure and explore helix formation in a systematic way.[35,36,40,41] Peptides of sequence $(AAAAK)_n$ have been shown to adopt structures whose helical content increases as n increases,[42] in agreement with established statistical models for helical formation and thermodynamic stability.[43,44] Since the lysine residues wrap completely around the outside of the α-helix after 4 sequence repeats, such peptides of $n > 4$ are strongly helical, very soluble, and positively charged, which keeps them from aggregating at neutral or acidic pH. Far-UV circular dichroism (CD) of amide electronic transitions has been used to show that these peptides undergo a clear, two-state unfolding transition from helix to random coil as the temperature increases:[35,36] the cooperativity of this transition increases as both n and the stability of the helical conformation increase. With N-terminal acetylation and C-terminal amidation (and thus complete amidation of the peptide backbone) these water-soluble peptides attain their maximum α-helical propensity via typical $(i, i + 4)$ hydrogen bonds between amide residues with no additional pairwise side chain interactions stabilizing the helix. With the goal of providing a more site-specific view of the structural ensemble, isotopic labeling studies[1,45,46] and time-resolved temperature jump experiments[47] were used to show that the N-terminus of N-acetylated alanine-rich peptides has both a higher helical content and a slower folding rate than the amide-capped C-terminus.

This study examines four peptides with $n = 5$ (see Table 1): a control peptide and three peptides with a single alanine-to-cyanylated cysteine substitution, each located at the same position vs lysine in a different polyalanine repeat unit. The stability of helical structure in these peptides (observed via far-UV CD) is used to determine the degree of disruption to the helix due to the probe side chain. The side chain's C≡N stretching absorption band is used to document its sensitivity to changes in the local secondary structure of the peptide.

Experimental Methods

Peptide Synthesis and Purification. All peptides were synthesized on an Applied Biosystems ABI 433A synthesizer using standard fmoc chemistry with 10× HATU activator. Fmoc-labeled amino acids and all peptide synthesis reagents (see below) were purchased from Applied BioSystems and used as received. The PAL resin was used to furnish a C-terminal carboxamide on cleavage; treatment of the resin bound peptides with acetic anhydride was used for N-acetylation. Cleaved peptides were purified via reversed-phase HPLC using a semiprep scale Dynamax C18 column. The eluent was 15−40% MeCN in H_2O (with 0.1% trifluoroacetic acid, TFA as modifier) over 45 min, and the last major peak was collected. Purity and identity of peptides was verified by MALDI-MS (performed at the Wistar Institute, Philadelphia, PA; see Supporting Information).

Cyanylation of Peptides. Lyophilized cysteine-containing peptide from HPLC was treated for 20 min in 0.01 M HCl solution and relyophilized to remove residual TFA. The peptide was weighed after lyophilization, dissolved in 50 mM phosphate buffer, pH 7.0−7.5, and treated with 100× D,L-dithiothreitol (DTT, from Aldrich) to ensure the free thiol. Residual DTT was separated from the peptide using a 8 cm column of Sephadex G-10, and the reduced peptide was lyophilized. The peptide was redissolved in 200 mM phosphate buffer, pH 6.5−7.0, and treated with 5× 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, from Acros) to form a mixed disulfide at cysteine. Absorbance of the sample was monitored at 412 nm to observe release of the thionitrobenzoate anion and quantify the yield of the mixed disulfide. The sample was then treated with 50× NaCN (Aldrich) and the cyanylated peptide was isolated using the same 8 cm Sephadex G-10 column equilibrated with 20 mM sodium phosphate buffer, pH 6.5−7.0. Peptide-containing fractions were concentrated about 5× using a Speed Vac centrifugal vacuum device, and the presence of the C≡N label was verified using infrared spectroscopy. Final concentrated sample concentration was 1−2 mM in 100−200 mM phosphate buffer, pH 6.5−7.0.

Far-UV Circular Dichroism. CD spectra were collected from 185−260 nm using an Aviv model 410 spectropolarimeter equipped with a temperature-controlled cell. Peptide samples at 1−2 mM peptide concentration (and 100−200 mM sodium phosphate buffer) were analyzed in a 10 μm quartz demountable cell (Starna Cells). No concentration dependence was observed for CD spectra in any of the peptides.

Temperature-dependent spectra were collected for samples at 10−20 μM peptide concentration (and 1−2 mM buffer, 100× dilutions of the original samples) in a 1 mm sealed quartz cell. After the sample temperature was raised, the sample was returned to 25 °C to verify reversibility of unfolding. Some minor irreversibility was observed when cyanylated samples were raised to 50 °C; any T below that led to completely reversible spectral changes.

Infrared Spectroscopy. Cyanylated peptide samples were placed between the windows of a 22 μm CaF_2 BioCell (BioTools, Jupiter, FL) placed inside a BioJack temperature-circulating jacket. Variable-temperature samples were returned to 25 °C after heating to verify reversibility of results: only samples raised above 45 °C showed evidence of irreversibility due to probe decomposition. All spectra were collected at 2 cm^{-1} resolution using a Bruker Optics Vertex 70 FTIR spectrometer with a photovoltaic HgCdTe detector. A spectrum of buffer solution at the appropriate temperature was subtracted from the raw spectrum, and further baseline correction was accomplished by fitting the baseline (outside the region from 2145−2185 cm^{-1}) to a polynomial and subtracting the fit.

C≡N stretching peaks were analyzed by fitting to a pseudo-Voigt profile that is the linear combination of a Gaussian and Lorentzian with the same width:

$$y_0 + A\left(m_{\mathrm{Lorentz}}\frac{2}{\pi}\frac{\mathrm{FWHM}}{4(\tilde{\nu}-\tilde{\nu}_c)^2+\mathrm{FWHM}^2} + (1-m_{\mathrm{Lorentz}})\frac{\sqrt{4\ln 2}}{\sqrt{\pi}\mathrm{FWHM}}\,\mathrm{e}^{-(4\ln 2/\mathrm{FWHM}^2)(\tilde{\nu}-\tilde{\nu}_c)^2}\right) \quad (1)$$

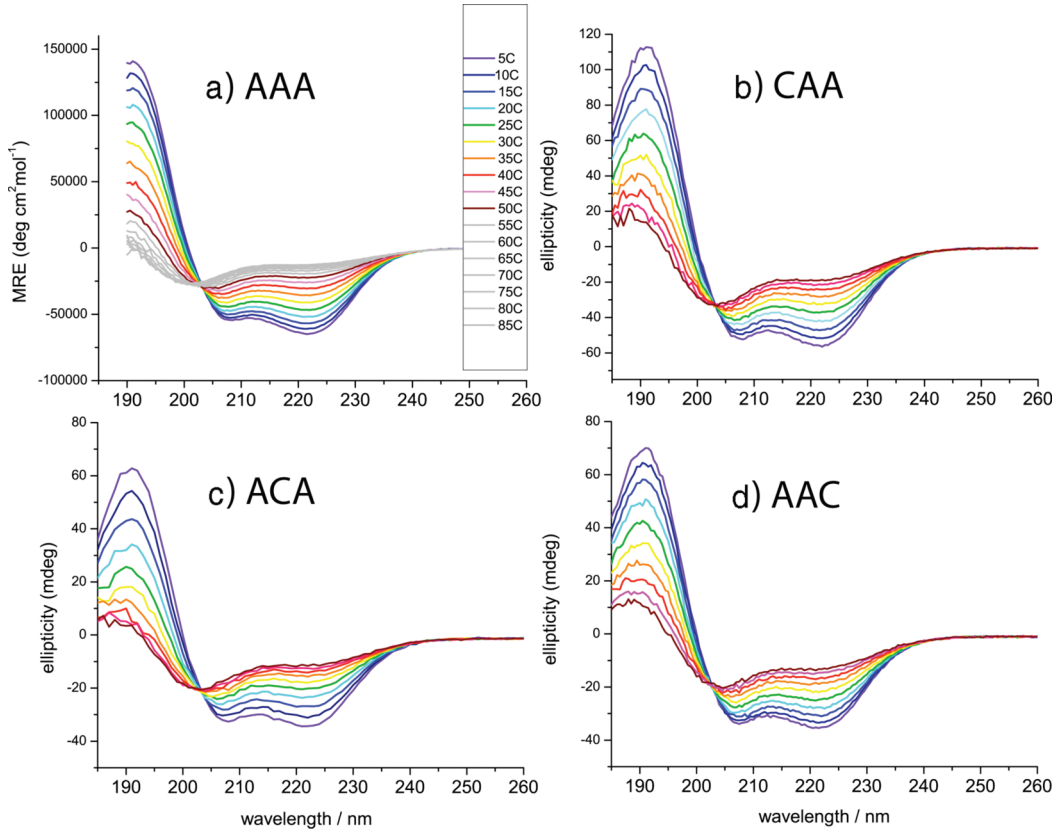

Figure 1. Temperature-dependent far-UV circular dichroism spectra for peptides (a) AAA, (b) CAA, (c) ACA, and (d) AAC.

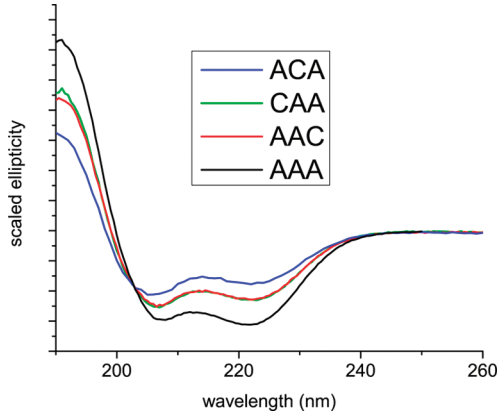

Figure 2. Far-UV circular dichroism spectra at 25 °C for all four peptides at approximately 1 mM concentration, with signals scaled to common intensity at the unfolding isosbestic point of 203 nm.

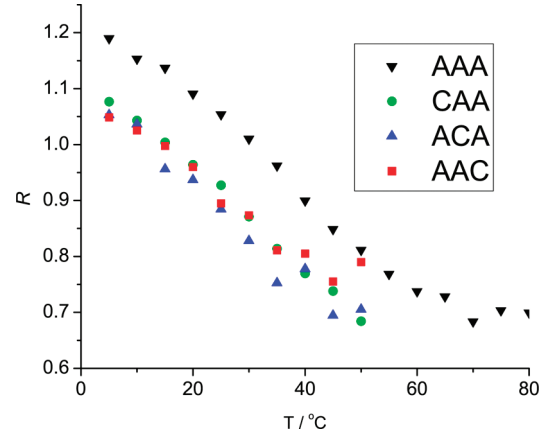

Figure 3. Helix melting curves for all four peptides, from data in Figure 1 using the empirical measure $R = \Theta_{222}/\Theta_{208}$.

This analysis assumes a symmetric peak and yields the single width parameter (FWHM) used for band comparisons.

Results and Discussion

Peptide Synthesis and Cyanylation. Alanine-repeat peptides are prone to deletion impurities: all peptides in this study required preparative HPLC purification (see Supporting Information for details), and yields were never higher than 50%. Once the cysteine-containing peptides were isolated, modification of the single cysteine residue for each peptide proceeded with quantitative yield. The cyanylated peptides are stable over the pH range of roughly 6.0−8.0, and they are stable for days at temperatures below 40 °C. Above 40 °C, the infrared signal from the SC≡N group is observed to decay slowly with time (on the time scale of several hours); above 50 °C this decyanylation process accelerates. Decomposition of the SCN-labeled peptide is likely via one of the pathways proposed previously in the literature, possibly accompanied by scission of the peptide backbone.[21,23,48] No further experiments were performed to identify decomposition products, and any sample with noticeably reduced C≡N band intensity was discarded. All spectra reported here are for intact cyanylated peptide samples.

Far-UV Circular Dichroism. Figure 1 shows temperature-dependent CD spectra for the four peptides, with the raw ellipticity signal for cyanylated peptides displayed in millidegrees. Each spectrum exhibits strong helical character at 5 °C, as evidenced by the two minima at ∼205 and ∼222 nm:[49] as the temperature increases, each peptide displays a clear two-state transition with an isosbestic point at 203 nm. This indicates that the qualitative folding behavior of all label-containing

peptides is identical to that of the unlabeled control peptide AAA: a clear helix-random coil transition is observed in all cases.

Figure 2 compares the CD spectrum at 25 °C for all four peptides. Since all sequences contain the same number of amide residues, and since each displays a helix−coil transition with isosbestic point at 203 nm, the spectra were scaled so that their intensities would match at 203 nm to account for any variations in concentration between samples. Using the spectral values at 208 and 222 nm as empirical markers of helical content,[46,50] it is clear that the extent of helicity varies in the order AAA > AAC = CAA > ACA. This indicates that the introduced β-thiocyanatoalanine residue has a destabilizing influence on the helical structure. Not surprisingly, placing the artifical side chain in the center repeat of the sequence is a greater perturbation than in either of the end repeats, whose perturbations are nearly identical. This result is in full agreement with widely cited statistical models for helix formation,[43,44] which place a greater thermodynamic emphasis on residues near the center of a sequence due to their ability to propagate the perturbation in either direction along the peptide chain. It is important to note, however, that the change in helical content in all three label-containing sequences is relatively minor. The basic helical propensity and helix−coil folding of the alanine-repeat sequence is largely preserved when cyanylated cysteine is introduced. Quantitative helical propensities have been shown to depend on peptide length and sequence, so a direct quantitative comparison to other amino acids substitutions is not warranted here: but the approximate melting points decrease by approximately 10, 15, and 10 °C for CAA, ACA, and AAC as compared to AAA, as shown in Figure 3 by comparison of temperature-dependent spectral R values[50] (where $R = \Theta_{222}/\Theta_{208}$ and greater R is interpreted as greater helical content). These data indicate that a single cyanylated cysteine side chain is not a fatal perturbation of secondary structure in sequences that already display a helical propensity.

Infrared Spectroscopy. Figure 4 shows the temperature-dependent C≡N stretching band of methyl thiocyanate in a buffer solution identical to that used for the peptide samples. As the temperature increases, the band systematically shifts to the red due to changes in the ability of water to interact with with the C≡N moiety. (The formation of hydrogen bonds to nitriles leads to a blue shift in the C≡N stretching frequency.[24,51,52]) No other clear changes in intensity or width are observed; line shape analysis indicates that the band narrows slightly with temperature, perhaps due to faster water dynamics and motional narrowing at higher temperatures.

Methyl thiocyanate was chosen as the "control" since it is highly water-soluble and contains the vibrational chromophore of interest without any possible effects from local structure other than water. Short, "unstructured" peptides were also considered as possible comparisons for the temperature dependence of the SC≡N band in water in the absence of a peptide folding process, but all peptide samples (even di- and tripeptides with capped ends) are expected to exhibit some variation in their structural distributions with temperature. An additional consideration is that many short peptides are not truly "unstructured" in solution, with one or more predominant conformations that might extend to discrete solvated structures, according to many recent experiments.[53−56]

Figure 5 presents the temperature-dependent infrared absorption spectra in the C≡N stretching region for the three modified peptides. Like the model compound's band in the absence of peptide, the C≡N stretching frequency shifts systematically to the red as the temperature increases. However, unlike the band for MeSC≡N, the line width narrows noticeably for all three label-modified peptides as the temperature increases. Fitted line widths for all of the spectra in Figures 4 and 5 are displayed in Figure 6. Far-UV CD indicates that the conformation of each peptide shifts from a more helical population at low T to one that is more disordered at high T. The C≡N stretching band's narrowing trend with temperature suggests that a labeled side chain attached to a helical secondary structure has a broader line width than the same side chain attached to a disordered backbone.

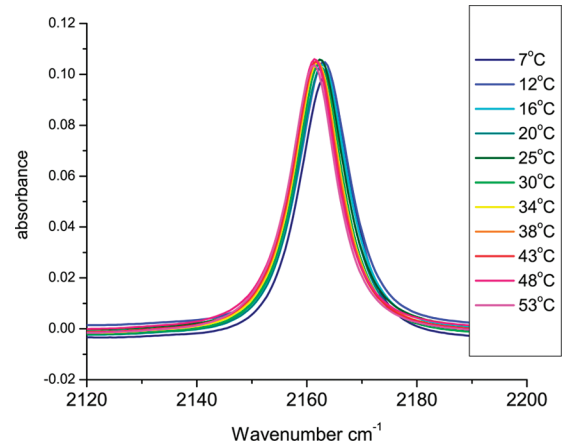

Figure 4. Temperature-dependent infrared absorption spectra in the CN stretching region for methyl thiocyanate in 150 mM phosphate buffer, pH 7.0.

Comparing the line widths from the three labeled side chains, they follow this approximate trend at all temperatures: CAA > AAC ≈ ACA. Despite the fact that the global secondary structures of CAA and AAC are nearly the same as indicated by CD spectra (Figure 2), the label near the C-terminal end of the peptide displays a narrower C≡N stretching band than the label near the N-terminal end. Previous labeling/vibrational CD[2] and T-jump[47] experiments indicate that the N-terminus of acetyl-capped alanine-repeat peptides is significantly more helical than the C-terminus. If a more helical local secondary structure leads to a broader C≡N line width, then our observation is consistent with (albeit more subtle and indirect than) the previous analysis of site-specific secondary structure. Since CD results indicate that the ACA peptide is globally less helical than either CAA or AAC, its side chain C≡N band is not expected to be substantially broader than that of CAA.

Temperature-dependent infrared spectra and line width analysis indicate that the cyanylated cysteine side chain is weakly, yet systematically, sensitive to local secondary structure in these peptides in the following way: greater local helical content leads to broader C≡N line width. There are two possible explanations for this result. One is that the increased line width could be due to the Stark effect of the helix dipole on the C≡N transition dipole. The SC≡N band frequency has been shown to be sensitive to local electrostatic forces in proteins.[14] Since the cyanylated cysteine side chain is free to rotate at the β-carbon, it should be able to adopt a range of different orientations vs a well-established helical structure. The CN stretching frequency should depend on the angle between the CN transition dipole and the helical dipole, with 0° leading to an opposite frequency shift from 180°. Thus the effect of the large helical dipole[57,58] on the C≡N stretching frequency could be that the local presence of a helix leads to a greater inhomogeneous distribution of C≡N stretching frequencies due to different orientations of

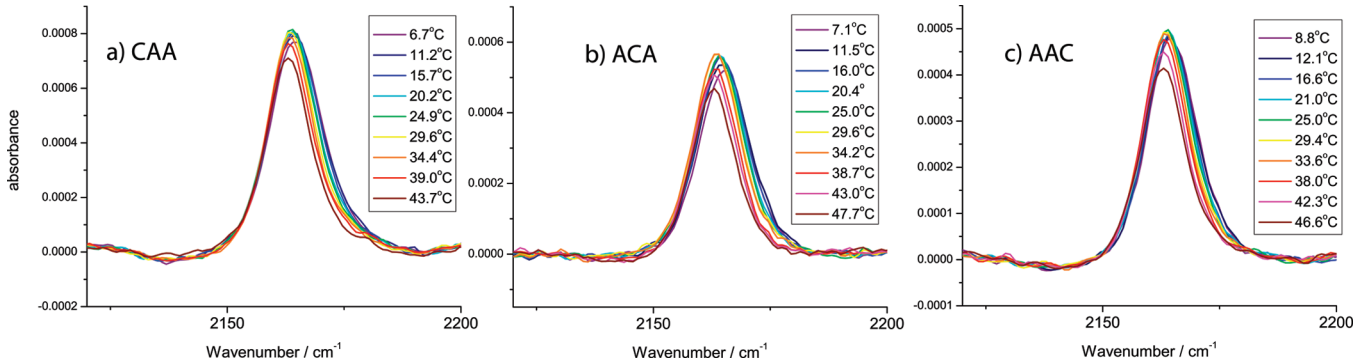

Figure 5. Temperature-dependent infrared absorption spectra in the CN stretching region for (a) CAA, (b) ACA, and (c) AAC.

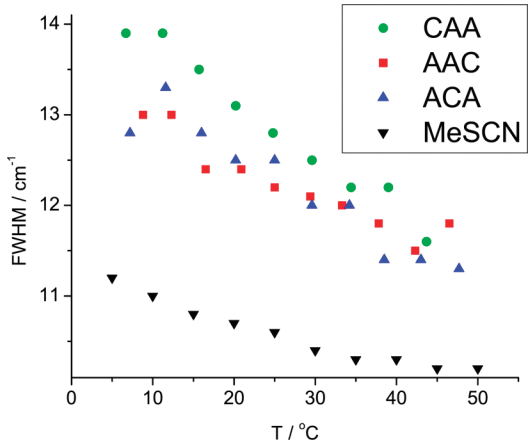

Figure 6. Full-width at half-maximum (FWHM) for the CN stretching bands of each cyanylated peptide, from fits to data in Figure 5.

the side chain with respect to the helical axis. Were the large helical dipole not present, the CN stretching frequency would not be modulated as much by varying Stark effects between the helix and the CN transition dipole and thus the line width would be narrower for a nonhelical peptide.

The alternate explanation for narrowing with decreased helical content is faster water dynamics. The line shape of the aliphatic SC≡N band has been shown to be sensitive to changes in picosecond solvent dynamics.[24] If the picosecond dipolar dynamics of water near the peptide are slower in the presence of a well-formed helix, then the C≡N stretching band (whose frequency has been indicated to be sensitive to the orientation of water molecules in the immediate vicinity[25,59]) should narrow with a decrease in the helical content of the local structure. Even if the inhomogeneous distribution of frequencies is the same for the solvent-exposed probe moiety, the speeding up of CN spectral diffusion by faster water motions would narrow the C≡N stretching band given standard models for spectral line shapes.[60,61] If this is the explanation for the line width changes in the probe vibration, it suggests somewhat provocatively that something as basic as a solvent-passivated helical structure is capable of substantially altering the picosecond dynamics of dipolar reorientation in water, which largely originate in formation and breaking of the aqueous hydrogen bond network.[62] This suggestion is interesting in light of recent work using time-resolved Stokes shifts of covalently attached dyes[63] or terahertz spectroscopy[64] to measure the picosecond dynamics of water near protein surfaces.

To distinguish between the two possible explanations for the C≡N band's narrowing with decreasing helical content, an experiment capable of measuring the spectral diffusion dynamics of the C≡N band[15] would be needed. A spectral observable related to the band's frequency autocorrelation function would reveal the dynamics of the band's frequency fluctuations. If the C≡N width changes were due to a helix-induced Stark effect, the spectral diffusion dynamics would remain unchanged regardless of secondary structure content. If the width changes were due to motional narrowing, the correlation function should change with helical content. A complementary experimental approach would be to investigate the cyanylated cysteine probe in the context of a β-sheet, which does not display the same unidirectional dipole as a helix and might lead to very different local Stark effects. But regardless of the origin of C≡N band narrowing, the infrared results presented here indicate that the C≡N band of cyanylated cysteine may be able to report local gain-of-structure transitions in proteins even when the probe moiety is solvent-exposed throughout the structural transition.

Conclusions

Using alanine-repeat peptides of established helical propensity, we have shown that cyanylated cysteine is not a strong disrupter of helical structure in solvent-exposed helices. In addition, the CN stretching band of the modified side chain is weakly sensitive to changes in the local secondary structure even when the side chain is solvent-exposed at all stages of a structural transition. Explaining this sensitivity, which comes through the CN line shape, could be an interesting challenge for nonlinear infrared experiments and simulation methods. Because it does not strongly influence the local structure, and since it is sensitive to local structural transitions, cyanylated cysteine could find wide use as a probe of site-specific structure in intrinsically disordered proteins or helix-forming subdomains of other biologically relevant protein systems.

Acknowledgment. The groups of Karin Akerfeldt and Robert Fairman, especially Bashkim Kokona, are acknowledged for assistance with peptide synthesis and purification. Sean Decatur is acknowledged for illuminating consultation and advice on peptide synthesis. This work was supported by Haverford College, a New Faculty Start-Up Award from the Dreyfus Foundation, a Cottrell College Science Award from the Research Corporation, and an AREA grant R15GM088749 from the National Institute of General Medical Sciences. This content is solely the responsibility of the authors and does not necessarily represent the official views of any of the funding agencies above.

Supporting Information Available: Peptide characterization, including HPLC purification of synthetic polymers, MALDI spectra for molecular weight, and analysis of cyanylated peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Decatur, S. M.; Antonic, J. *J. Am. Chem. Soc.* **1999**, *121*, 11914–11915.
(2) Silva, R.; Kubelka, J.; Bour, P.; Decatur, S. M.; Keiderling, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8318–8323.
(3) Huang, R.; Kubelka, J.; Barber-Armstrong, W.; Silva, R. A. G. D.; Decatur, S. M.; Keiderling, T. A. *J. Am. Chem. Soc.* **2004**, *126*, 2346–2354.
(4) Setnicka, V.; Huang, R.; Thomas, C. L.; Etienne, M. A.; Kubelka, J.; Hammer, R. P.; Keiderling, T. A. *J. Am. Chem. Soc.* **2005**, *127*, 4992–4993.
(5) Brewer, S. H.; Song, B. B.; Raleigh, D. P.; Dyer, R. B. *Biochemistry* **2007**, *46*, 3279–3285.
(6) Chin, J. K.; Jimenez, R.; Romesberg, F. E. *J. Am. Chem. Soc.* **2001**, *123*, 2426–2427.
(7) Cremeens, M. E.; Fujisaki, H.; Zhang, Y.; Zimmermann, J.; Sagle, L. B.; Matsuda, S.; Dawson, P. E.; Straub, J. E.; Romesberg, F. E. *J. Am. Chem. Soc.* **2006**, *128*, 6028–6029.
(8) Sagle, L. B.; Zimmermann, J.; Dawson, P. E.; Romesberg, F. E. *J. Am. Chem. Soc.* **2004**, *126*, 3384–3385.
(9) Thielges, M. C.; Case, D. A.; Romesberg, F. E. *J. Am. Chem. Soc.* **2008**, *130*, 6597–6603.
(10) Lindquist, B. A.; Furse, K. E.; Corcelli, S. A. *Phys. Chem. Chem. Phys.* **2009**, *11*, 8119–8132.
(11) Getahun, Z.; Huang, C. Y.; Wang, T.; De Leon, B.; DeGrado, W. F.; Gai, F. *J. Am. Chem. Soc.* **2003**, *125*, 405–411.
(12) Tucker, M. J.; Getahun, Z.; Nanda, V.; DeGrado, W. F.; Gai, F. *J. Am. Chem. Soc.* **2004**, *126*, 5078–5079.
(13) Waegele, M. M.; Tucker, M. J.; Gai, F. *Chem. Phys. Lett.* **2009**, *478*, 249–253.
(14) Fafarman, A. T.; Webb, L. J.; Chuang, J. I.; Boxer, S. G. *J. Am. Chem. Soc.* **2006**, *128*, 13356–13357.
(15) Fang, C.; Bauman, J. D.; Das, K.; Remorino, A.; Arnold, E.; Hochstrasser, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 1472–1477.
(16) Webb, L. J.; Boxer, S. G. *Biochemistry* **2008**, *47*, 1588–1598.
(17) Schultz, K. C.; Supekova, L.; Ryu, Y. H.; Xie, J. M.; Perera, R.; Schultz, P. G. *J. Am. Chem. Soc.* **2006**, *128*, 13984–13985.
(18) Taskent-Sezgin, H.; Chung, J.; Patsalo, V.; Miyake-Stoner, S. J.; Miller, A. M.; Brewer, S. H.; Mehl, R. A.; Green, D. F.; Raleigh, D. P.; Carrico, I. *Biochemistry* **2009**, *48*, 9040–9046.
(19) Tucker, M. J.; Kim, Y. S.; Hochstrasser, R. M. *Chem. Phys. Lett.* **2009**, *470*, 80–84.
(20) Degani, Y.; Degani, C. *Biochemistry* **1979**, *18*, 5917–5923.
(21) Degani, Y.; Neumann, H.; Patchornik, A. *J. Am. Chem. Soc.* **1970**, *92*, 6969–6971.
(22) Degani, Y.; Patchornik, A. *Biochemistry* **1974**, *13*, 1–11.
(23) Doherty, G. M.; Motherway, R.; Mayhew, S. G.; Malthouse, J. P. *Biochemistry* **1992**, *31*, 7922–7930.
(24) Maienschein-Cline, M. G.; Londergan, C. H. *J. Phys. Chem. A* **2007**, *111*, 10020–10025.
(25) Choi, J. H.; Oh, K. I.; Lee, H.; Lee, C.; Cho, M. *J. Chem. Phys.* **2008**, *128*, 134506.
(26) Radivojac, P.; Iakoucheva, L. M.; Oldfield, C. J.; Obradovic, Z.; Uversky, V. N.; Dunker, A. K. *Biophys. J.* **2007**, *92*, 1439–1456.
(27) Dunker, A. K.; Obradovic, Z. *Nat. Biotechnol.* **2001**, *19*, 805–806.
(28) Dyson, H. J.; Wright, P. E. *Curr. Opin. Struct. Biol.* **2002**, *12*, 54–60.
(29) Fuxreiter, M.; Simon, I.; Friedrich, P.; Tompa, P. *J. Mol. Biol.* **2004**, *338*, 1015–1026.
(30) Uversky, V. N.; Oldfield, C. J.; Dunker, A. K. *J. Mol. Recog.* **2005**, *18*, 343–384.
(31) Morin, B.; Bourhis, J. M.; Belle, V.; Woudstra, M.; Carriere, F.; Guigliarelli, B.; Fournel, A.; Longhi, S. *J. Phys. Chem. B* **2006**, *110*, 20596–20608.
(32) Columbus, L.; Hubbell, W. L. *Trends Biochem. Sci.* **2002**, *27*, 288–295.
(33) Belle, V.; Rouger, S.; Costanzo, S.; Liquière, E.; Strancar, J.; Guigliarelli, B.; Fournel, A.; Longhi, S. *Proteins: Struct. Funct. Bioinf.* **2008**, *73*, 973–988.
(34) Langen, R.; Oh, K. J.; Cascio, D.; Hubbell, W. L. *Biochemistry* **2000**, *39*, 8396–8405.
(35) Marqusee, S.; Robbins, V. H.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5286–5290.
(36) Chakrabartty, A; Kortemme, T.; Baldwin, R. *Protein Sci.* **1994**, *3*, 843–852.
(37) Fersht, A. *Structure and Mechanism in Protein Science*; W. H. Freeman: New York, NY, 1999.
(38) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2833–2837.
(39) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Biochemistry* **1997**, *36*, 10923–10929.
(40) Chakrabartty, A.; Doig, A. J.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11332–11336.
(41) Doig, A. J.; Baldwin, R. L. *Protein Sci.* **1995**, *4*, 1325–1336.
(42) Wang, T.; Zhu, Y. J.; Getahun, Z.; Du, D. G.; Huang, C. Y.; DeGrado, W. F.; Gai, F. *J. Phys. Chem. B* **2004**, *108*, 15301–15310.
(43) Lifson, S.; Roig, A. *J. Chem. Phys.* **1961**, *34*, 365–368.
(44) Zimm, B. H.; Bragg, J. K. *J. Chem. Phys.* **1959**, *31*, 526–535.
(45) Barber-Armstrong, W.; Donaldson, T.; Wijesooriya, H.; Silva, R.; Decatur, S. M. *J. Am. Chem. Soc.* **2004**, *126*, 2339–2345.
(46) Silva, R.; Nguyen, J. Y.; Decatur, S. M. *Biochemistry* **2002**, *41*, 15296–15303.
(47) Ramajo, A. P.; Petty, S. A.; Starzyk, A.; Decatur, S. M.; Volk, M. *J. Am. Chem. Soc.* **2005**, *127*, 13784–13785.
(48) Degani, Y.; Patchornik, A.; Maclaren, J. A. *J. Am. Chem. Soc.* **1966**, *88*, 3460–3461.
(49) Woody, R. W. *Monatsh. Chem.* **2005**, *136*, 347–366.
(50) Sudha, T. S.; Vijayakumar, E. K. S.; Balaram, P. *Int. J. Pept. Protein Res.* **1983**, *22*, 464–468.
(51) Cha, J. N.; Cheong, B. S.; Cho, H. G. *J. Mol. Struct.* **2001**, *570*, 97–107.
(52) Huang, C. Y.; Wang, T.; Gai, F. *Chem. Phys. Lett.* **2003**, *371*, 731–738.
(53) Eker, F.; Griebenow, K.; Cao, X. L.; Nafie, L. A.; Schweitzer-Stenner, R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10054–10059.
(54) Grdadolnik, J.; Grdadolnik, S. G.; Avbelj, F. *J. Phys. Chem. B* **2008**, *112*, 2712–2718.
(55) Kim, Y. S.; Hochstrasser, R. M. *J. Phys. Chem. B* **2007**, *111*, 9697–9701.
(56) Shi, Z. S.; Chen, K.; Liu, Z. G.; Kallenbach, N. R. *Chem. Rev.* **2006**, *106*, 1877–1897.
(57) Lockhart, D. J.; Kim, P. S. *Science* **1992**, *257*, 947–951.
(58) Sengupta, D.; Behera, R. N.; Smith, J. C.; Ullmann, G. M. *Structure* **2005**, *13*, 849–855.
(59) Oh, K. I.; Choi, J. H.; Lee, J. H.; Han, J. B.; Lee, H.; Cho, M. *J. Chem. Phys.* **2008**, *128*, 154504.
(60) Kubo, R. *Adv. Chem. Phys.* **1969**, *15*, 101–127.
(61) Skinner, J. L. *Mol. Phys.* **2008**, *106*, 2245–2253.
(62) Skinner, J. L.; Auer, B. M.; Lin, Y. S. *Adv. Chem. Phys.* **2009**, *142*, 59–103.
(63) Qiu, W. H.; Kao, Y. T.; Zhang, L. Y.; Yang, Y.; Wang, L. J.; Stites, W. E.; Zhong, D. P.; Zewail, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13979–13984.
(64) Ebbinghaus, S.; Kim, S. J.; Heyden, M.; Yu, X.; Heugen, U.; Gruebele, M.; Leitner, D. M.; Havenith, M. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 20749–20752.

JP101447R