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Infrared Spectra of Amide Groups in α-Helical Proteins: Evidence for Hydrogen Bonding between Helices and Water

Eric S. Manas,† Zelleka Getahun, Wayne W. Wright, William F. DeGrado, and Jane M. Vanderkooi*

Contribution from the Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6089

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Abstract: Infrared spectral frequencies of amide vibrational modes are sensitive to secondary structure. In this work, evidence is presented that accessibility to water additionally affects spectral positions. The dimeric α-helical coiled-coil GCN4-P1′ was ^{13}C labeled in the amide carbonyl groups of buried Leu or exposed Ala. At 20 °C, the amide I′ peak for ^{13}C Ala amide is at 1585 cm^{-1}, whereas the position for ^{13}C Leu is at 1606 cm^{-1}. These shifts permit the distinction of solvent-exposed and buried amide groups. Lowering temperature increases H-bond strength, producing a shift to lower frequency. In the temperature range from 10 to 273 K in aqueous glycerol, the amide transitions assigned to solvent-exposed regions of the helices undergo the strongest temperature-dependent shifts, similar to that of the peptide bond model compound, *N*-methylacetamide, in the same aqueous solvent. In addition, spectral shifts of the amide bands for *N*-methylacetamide and the solvent-exposed component of the proteins follow the glass transition temperature of the cryosolvent. In contrast, the amide transitions assigned to α-helical segments that are expected to have little interaction with water undergo the weakest shifts. The amide I′ band of the α-helical protein parvalbumin also shows subpeaks that shift differently with temperature, and on the basis of their temperature dependence and frequency can be assigned to solvent exposed or buried regions. The spectral shifts are discussed in terms of changes in hydrogen bond strengths, including contributions from volume expansion of the sample, and variations in the average hydrogen bond angle, induced by population of low-frequency librational modes involving the solvent and protein. The results on the isotopically labeled peptides conclusively show that α-helical regions that are or are not solvent exposed can be distinguished both by the position of the amide I′ peak and by the temperature-dependent shifts.

Introduction

The folding of a one-dimensional polypeptide chain into a three-dimensional protein with unique structure depends on the interplay of forces exerted between neighboring groups within the protein, as well as the interactions between the protein and the surrounding medium.[1] Hydrogen bonding is an important governing force that influences secondary structure in proteins and specificity of folding.[2] Hydrogen bonding also has a profound effect on IR absorption lines, changing both the frequency of the transition and the extinction coefficient, due to a modification of the force constant of a given normal mode.[3]

* Author to whom correspondence should be addressed. Phone: 215-898-8783. E-mail: vanderko@mail.med.upenn.edu.
† Present address: Wyeth-Ayerst Research, 145 King of Prussia Road, Radnor, PA 19087.

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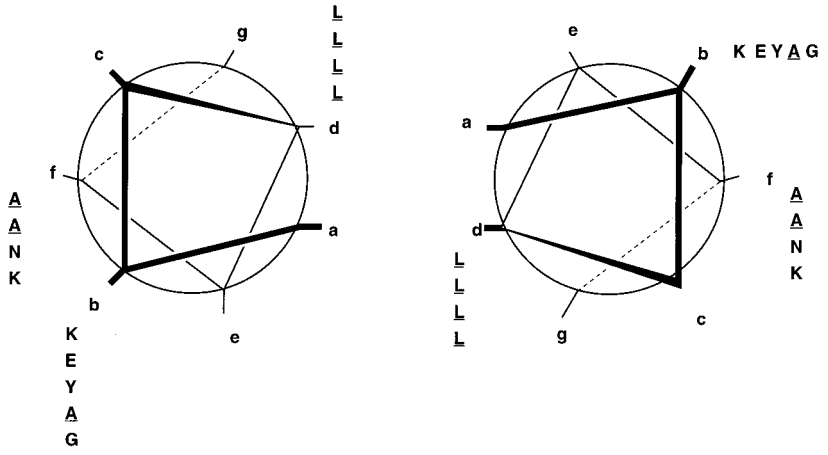

d f d f d b d

CH_3CONHR-M K Q L E A K- V E E L L A K -N Y H L E N E - VA R L K K L -V-G E R $CONH_2$ GCN4-P1'

CH_3CONHR-M K Q L E **A** K- V E E L L **A** K -N Y H L E N E - V**A** R L K K L -V-G E R $CONH_2$ ^{13}C-Ala-GCN4-P1'

CH_3CONHR-M K Q **L** E A K- V E E **L** L A K-N Y H **L** E N E - VA R **L** K K L -V-G E R $CONH_2$ ^{13}C-Leu-GCN4-P1'

Residues with ^{13}C label on the amide carbonyl are underlined

Figure 1. Sequence and positions of amino acids in GCN4-P1′, ^{13}Ala GCN4-P1′, and ^{13}Leu GCN4−P1′.

For amide groups, hydrogen bonding has long been recognized to influence IR bands.[4] Amide peak positions help to identify α and *β* helices in proteins.[5−8] The secondary structure assignment procedures based on IR generally use a single frequency as "α-helical".[9] However, recent work on solvent-exposed α helices shows IR frequencies that are different from those observed for solvent-inaccessible helices.[10−12] Indeed, α-helical coiled-coil proteins have been shown to have multiple frequencies that may arise from either differences in the conformation of different regions of the helices or differences in solvent exposure.[13−15]

To address this issue we examine the spectra of the dimeric α-helical coiled-coil GCN4-P1′. This synthetic peptide, from the 2-stranded coiled-coil of GCN4, is a frequently studied model whose X-ray and NMR structures have been intensively investigated.[16−22] In our study, we are interested in the influence of the amide environment on the observed IR amide frequencies. Two isotopic versions of this peptide were prepared, one in which the solvent-exposed carbonyl carbons of its amide Ala residues were labeled with ^{13}C (^{13}C-Ala-GCN4-P1′), and the second in which the Leu residues at each geometrically equivalent buried "d" position (^{13}C-Leu-GCN4-P1′) were similarly labeled. The characteristics of these peptides were compared with the unlabeled peptide. The sequences of the peptides studied and a depiction of their location in the helix are given in Figure 1. Since the frequency of the amide I′ band shifts with isotope labeling,[23,24] the absorbance of groups at different helix locations with different solvent exposure can be monitored independently from the broad absorption band of the other amide groups. *N*-Methylacetamide (NMA) serves as a model for a solvent-exposed peptide in our study. We also examine the temperature dependence of the infrared spectra for amide groups in the α-helical protein parvalbumin, which is approximately 40% helical.

It is well-known that temperature affects H-bonding and that IR bands of H-bonded groups are temperature dependent.[3] In proteins the amide I (I′) band tends to shift to higher frequency, while the amide II (II′) band tends to shift to lower frequency with increasing temperature, due to weakening of hydrogen bonds. Demmel and co-workers have utilized this feature to examine the temperature dependence of hydrogen bonding in myoglobin.[25] Over a temperature range of 80 to 300 K they found that the expected temperature dependence was observed. Further, they observed a discontinuity in the temperature dependence of the amide bands near the glass transition of the solvent. These authors suggested that hydrogen bonds in a well-defined α-helix would exhibit weaker temperature dependence than those between disordered regions of the protein and the solvent. However, this remains to be shown experimentally.

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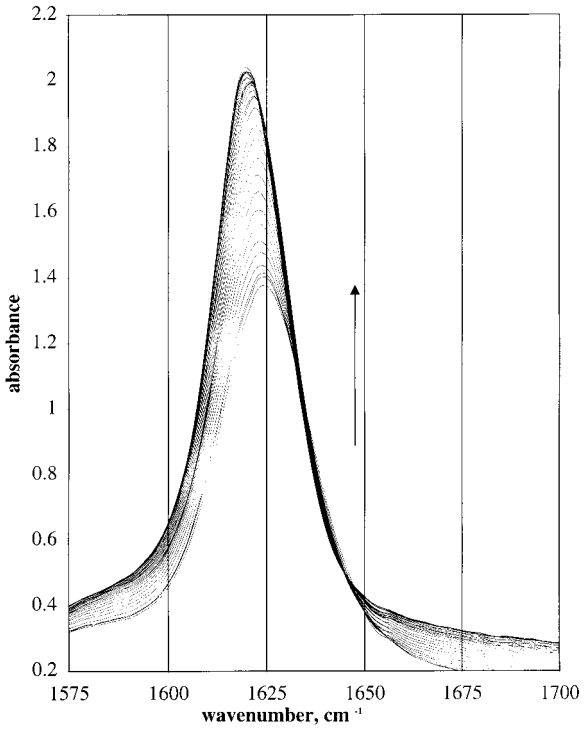

Figure 2. IR absorption spectra of the amide I′ region of NMA in D_2O-D_8-glycerol (50/50). The pD was ~7.0. The temperature was varied from 290 to 10 K in the direction of the arrow.

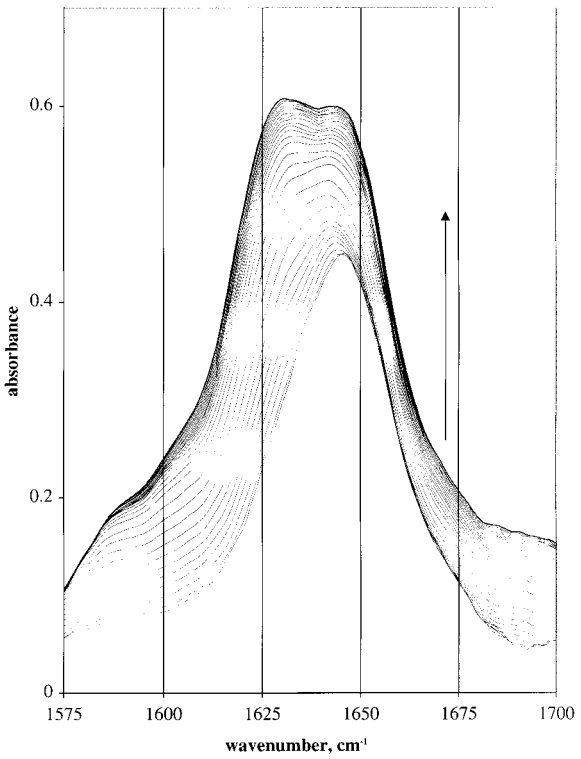

Figure 3. IR absorption spectra of GCN4-P1′. The conditions are the same as those given in Figure 2.

The purpose of our study is to examine whether the difference in resonance frequency can be attributed to different environments. We will show that amide subpeaks shift differently with temperature in the range 10 to 273 K. For instance, using ^{13}C labeling, the subpeaks of the amide I′ band attributed to solvent exposed regions shift more prominently with temperature, similar to the amide I′ band of NMA. In comparison, subpeaks attributed to α-helical regions not involved in solvent bonding shift very weakly with temperature, similar to the main amide I′ bands of GCN4.

Results

Temperature Dependence of the Infrared Spectra of NMA. Figure 2 shows the amide I′ peak of NMA in D_2O/D_8 glycerol (perdeuterated glycerol) in a temperature range of 300−10 K. The amide I′ peak of NMA, appearing with a maximum at 1625 cm^{-1} at 20 °C, shifts to lower frequency with decreasing temperature. The total amide I′ shift in going from room temperature to 10 K is -4.8 cm^{-1}. It can be noted also that as the temperature decreases the amide I′ peak absorption increases and the band becomes sharper. The amide II′ peak of NMA is a doublet with peaks at 1494 and 1514 cm^{-1} (not shown). The lower energy component of amide II′ has been attributed to Fermi resonance with the combination band at $873 + 632 = 1505$ cm^{-1}.[26] The amide II′ peaks shift to higher frequency with decreasing temperature, consistent with increasing hydrogen bond strength. The total amide II′ shift is $+6.7$ cm^{-1}.

IR Spectra of Isotopically Labeled α-Helical Coiled-Coils. The amide I′ spectra of the synthetic α-helical model protein GCN4-P1′ in D_2O/D_8 glycerol are shown as a function of temperature in Figure 3. The amide I′ band of GCN4-P1′ is broader than that observed in the model compound NMA. As was the case with NMA, the amide I′ band shifts to lower frequency with decreasing temperature, but as the temperature is lowered the spectrum also shows increasing structure. In particular, the amide I′ band appears to contain a component that does not shift with temperature at around 1650 cm^{-1} and a lower frequency component that becomes resolved and downshifts as the temperature is lowered. This suggests that the lower frequency component can be attributed to peptide bonds existing in a distinctly different environment.

GCN4 is a dimeric coiled-coil, with no β-turns or random coil segments per se. We considered the possibility that the lower frequency component can be assigned to segments or regions that are most involved in direct hydrogen bonding with the solvent, as was previously suggested by theoretical studies for the unusually low-frequency amide I′ band of coiled-coils.[14] This is supported by the fact that the component appears near the spectral region of the amide I′ band of NMA, which contains a solvent exposed peptide bond, and that it follows a similar temperature profile (see Figure 2). To test the idea that the amide groups of solvent exposed and buried groups absorb at different positions and exhibit different temperature dependence, the amide carbonyls were labeled with ^{13}C in the positions indicated in Figure 1.

The resulting absorption spectra are given in Figure 4 for ^{13}C-Ala-GCN4-P1′. Since the Ala carbonyl group is solvent exposed, the isotope shift from this residue would represent groups that are affected by the solvent. A peak at 1585 cm^{-1} is apparent at low temperature and can be identified as arising from the amide I′ stretch from the isotopic labeled amide group (Figure 4). At higher temperatures this peak becomes broad, and it also shifts to higher frequency. The broadening is larger than that for the model compound, NMA (Figure 2), so there is likely also to be additional environmental effects on the position. Comparing the spectrum for ^{13}C-Ala-GCN4-P1′ (Figure 4) with that of the unlabeled protein (Figure 3) one can see that the

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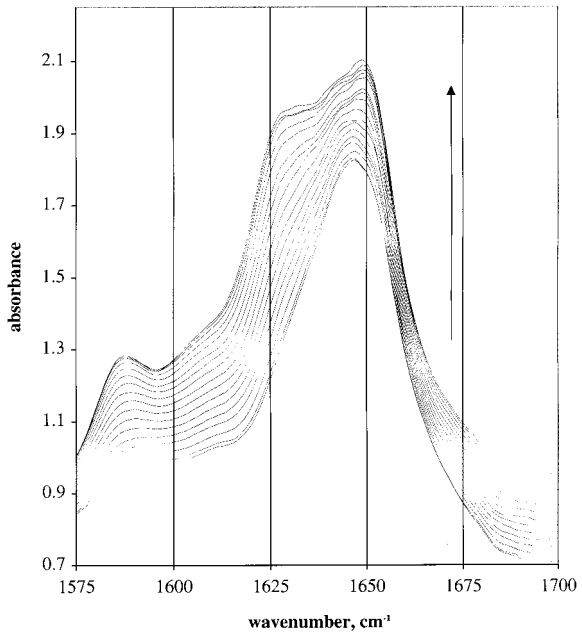

Figure 4. IR absorption spectra of ^{13}C Ala GCN4-P1′ in the amide I′ region. The conditions are the same as those given in Figure 2.

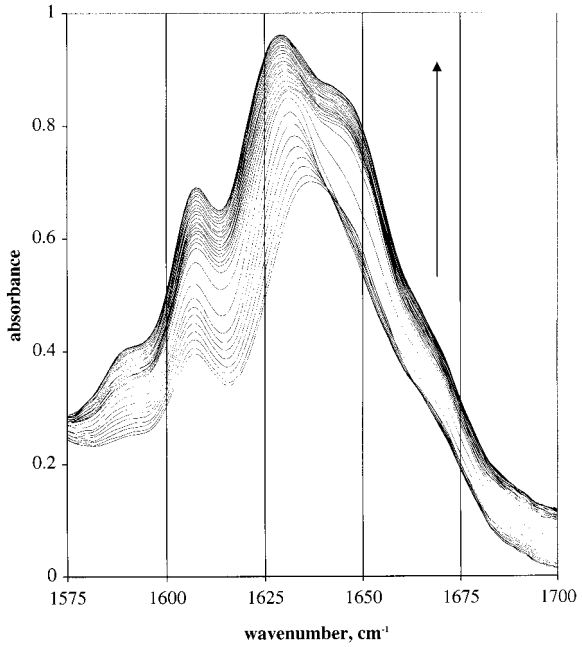

Figure 5. IR absorption spectra of ^{13}C Leu GCN4-P1′ in the amide I′ region. The conditions are the same as those given in Figure 2.

relative contribution of the peak at 1625 cm^{-1} is decreased in the labeled protein. Since some of the exposed amide groups are now ^{13}C labeled, the observation that the peak intensity decreases is consistent with the premise that absorption in this region arises from solvent H-bonded amide groups. The isotope shift is therefore 35−40 cm^{-1} in the helix. This is close to the observed isotope shift of 37 cm^{-1} for a carbonyl in model peptides.[23]

Spectra of the peptide in which the amide of Leu is ^{13}C labeled are shown in Figure 5. A peak at 1611 cm^{-1} is identified as arising from the isotopic labeled amide group. The maximum remains at the same frequency at all temperatures. The temperature dependence of the amide I′ region between 1625 and 1650 cm^{-1} can also be remarked upon. In the spectrum the peak that is attributed to the solvent-bonded amide groups becomes quite prominent at low temperature, since the relative absorbance contribution at 1648 cm^{-1} becomes less, because 8 residues of Leu are labeled by ^{13}C per dimer.

Analysis of the Spectra. To give a more quantitative assessment of the contribution to the amide I′ band from different protein environments, the amide I′ regions for the three GCN4 peptides were analyzed for their relative spectral contributions over the entire temperature range studied. The amide I′ band of GCN4-P1′ was successfully fit to two major Gaussian functions (Figure 6a for the sample at 110 K), although there is an indication of a contribution at higher frequency and less intense additional bands at lower frequency. As was suggested by visual inspection of the GCN4-P1′ spectra, the amide I′ band consists of at least two components or subpeaks that shift differently with temperature. The component at around 1650 cm^{-1} is typically attributed to the α-helical region of the protein.[7,27] Similar fits were obtained for ^{13}C-Ala-GCN4-P1′ (not shown) and for ^{13}C-Leu-GCN4-P1′ (Figure 6b), the latter showing the extra peak attributed to the isotopically labeled amide group.

The fits showed that peak position and intensity of the absorption band associated with the isotope change with temperature. Table 1 gives the peak positions and absorbance at the highest and lowest temperatures and these data for the entire temperature range are summarized in Figure 7a for the position and Figure 7b for the relative absorbance (the absorbance at a given temperature was divided by the absorbance at 290 K). The relative absorbance at 3500 cm^{-1} is also shown in Figure 7b. The peak position of the OH stretch frequency of the solvent is 3400 cm^{-1} at 290 K, and since this entire band shifts to lower frequency as the temperature decreases, the absorbance at 3500 cm^{-1} decreases as temperature decreases. The frequency at 3500 cm^{-1} was then taken to monitor the solvent glass transition, which for glycerol/water solvent occurred at 160−180 K. As seen in Figure 7a, the position change of the amide of NMA and exposed ^{13}C-Ala-GCN4-P1′ shows inflections at the glass transition. The position change of the isotope amide absorption in ^{13}C-Leu-GCN4-P1′, in contrast, had little change with temperature. The temperature dependence of the spectral shift and absorbance is strongest above the solvent glass transition. As a practical matter, for these, as well as all other spectra presented in this paper, no significant improvement in resolution is gained by decreasing the temperature below approximately 100 K. Spectral changes could still be seen over the low-temperature range, however, indicating that even at the lowest temperatures there are low-frequency modes that couple into the transitions.

Temperature Dependence of the Infrared Spectra of an α-Helical Protein. IR spectra of the amide I′ band for parvalbumin in D_2O/glycerol are shown as a function of temperature in Figure 8. The spectrum at room temperature resembles those previously reported.[29,30] The spectra are similar to those obtained for GCN4-P1′ in that the amide I′ bands of the protein and the peptide are broader than was observed for NMA. In addition, as the temperature is lowered, the spectra show increasing structure,and the amide I′ peaks shift to lower frequency, again consistent with the temperature dependence of hydrogen bonding. Also, a shoulder on the low-frequency

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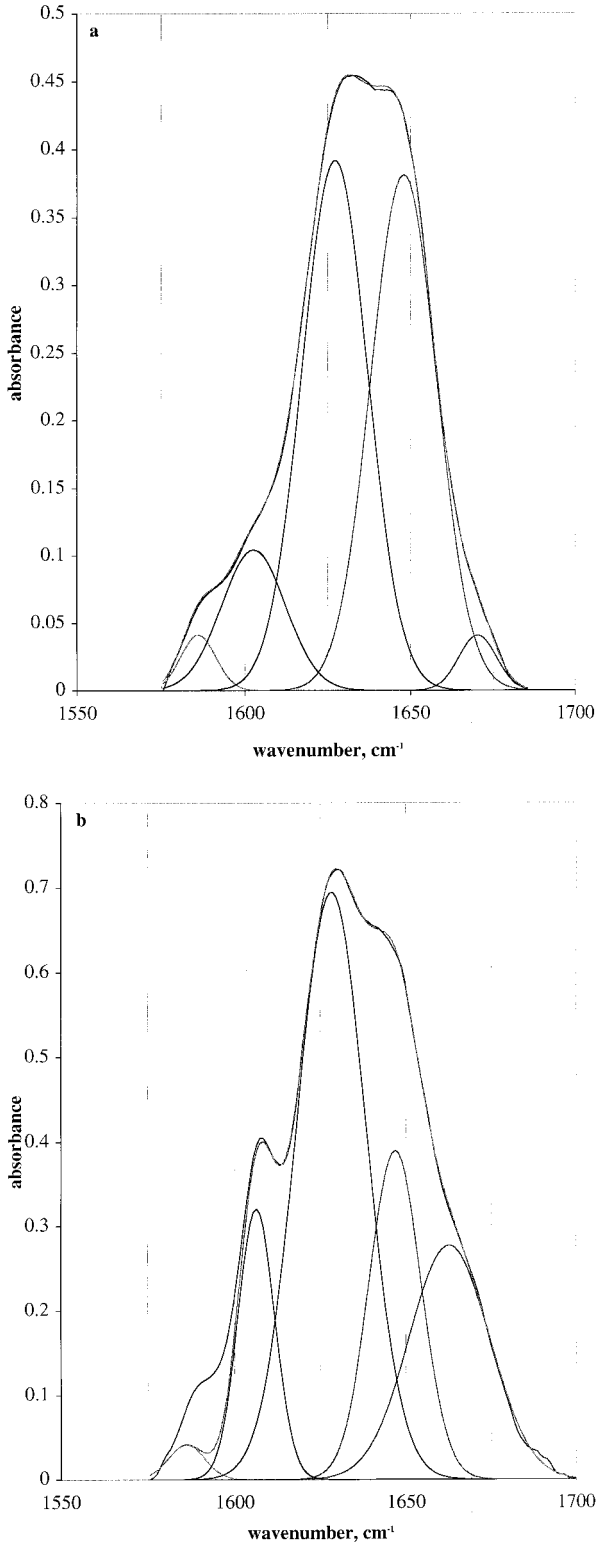

Figure 6. Example of deconvolution of peaks. The temperature was 110 K. (a) GCN4-P1′: Gaussian function fits had the following respective positions, widths in cm^{-1}, and relative amplitudes: 1586, 5.2, 0.04; 1603, 9.6, 0.1; 1627, 9.9, 0.39; 1648, 10, 0.38; and 1670, 5.6, 0.04. (b) ^{13}Leu-GCN4-P1′: Gaussian function fits had the following respective positions, widths in cm^{-1}, and relative amplitudes: 1591, 6.4, 0.11; 1606, 5.3, 0.32; 1628, 10.1, 0.69; 1647, 7.6, 0.39; and 1663, 11.7, 0.28.

side of the amide I′ band for both appears to grow in as the temperature is lowered.

We also deconvolved the amide I′ region for parvalbumin over the entire temperature range studied. As was suggested

Table 1. Peak Positions, Widths, and Absorbance of Isotopically Shifted Amide I′ in ^{13}Ala GCN4-−P1′ and ^{13}Leu GCN4-P1′

	temp, K	peak center, cm^{-1}	width, cm^{-1}	absorbance[a]
^{13}C Ala GCN4-P1′	290	1586.9	19.4	1
	13	1585.5	14.3	2.35
^{13}C Leu GCN4-P1′	290	1606.8	14.1	1
	13	1606.0	12.0	1.69

[a] Absorbance is relative to 290 K.

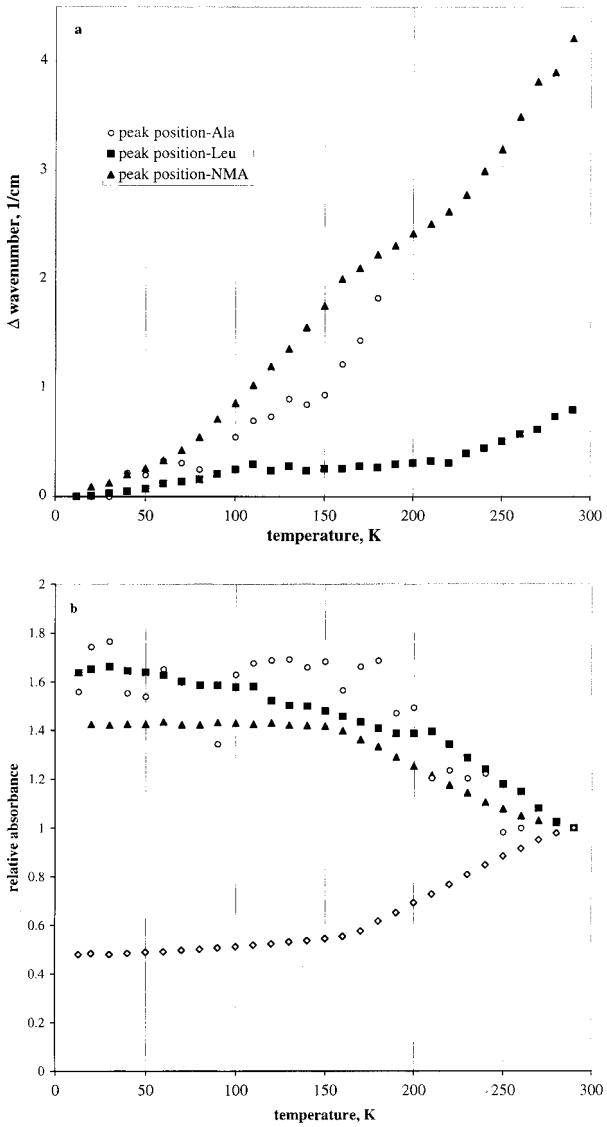

Figure 7. Peak position (a) and relative absorbance (b) of the peak attributed to amide I′ for the isotopically labeled amide group compared with amide I′ of NMA as a function of temperature. Symbols are identified on the figure; diamond is the relative absorbance at 3500 cm^{-1}. Due to the large noise in the data, arising from low absorbance and broadness of the peak, the value of the peak position for ^{13}C-Ala above 180 K was omitted.

by visual inspection of the GCN4-P1′ spectra, the protein amide I′ bands consist of components or subpeaks that shift differently with temperature. Again, the component at around 1650 cm^{-1} can be attributed to the α-helical region of the protein.[7,27] The higher frequency component at 1665−1670 cm^{-1} can be assigned to turns or internal random coil segments not involved in hydrogen bonding, which is consistent with the fact that this component accounts for less than 20% of the total peak area. This higher frequency component does not appear in the spectra

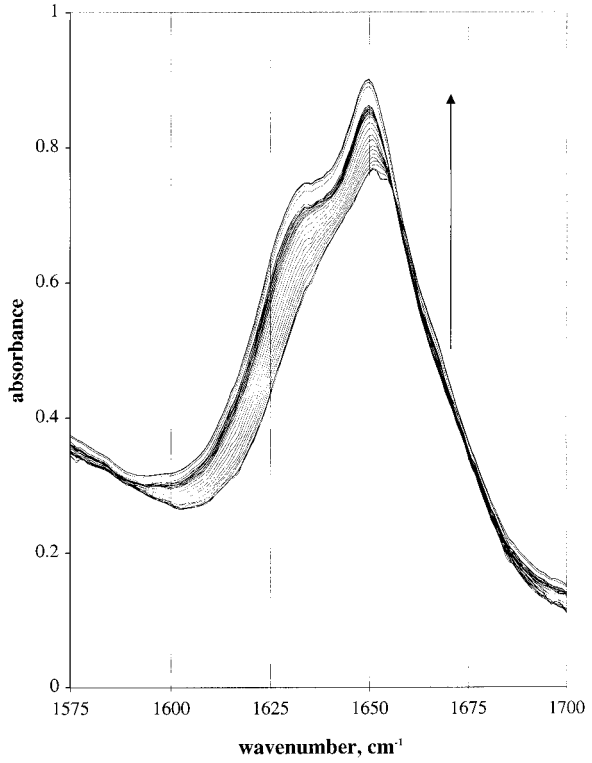

Figure 8. IR absorption spectra of parvalbumin in the amide I′ region. The conditions are the same as those given in Figure 2.

of GCN4-P1′, since it has no β-turns. On the other hand, it is also possible that this component contains contributions from distorted helices.[14]

We attribute the lower frequency component to groups that are most involved in direct hydrogen bonding with the solvent. The percentage area of this component is larger in parvalbumin than in the peptide; parvalbumin, having a greater amount of random coil segments, is likely to have a greater degree of hydrogen bonding with the solvent. In addition, the lower frequency component consistently undergoes the largest frequency shift upon raising or lowering the temperature, similar to NMA, which has a solvent exposed peptide bond. This effect is demonstrated in Figure 9, which shows the helical and solvent exposed amide I′ peak frequencies as a function of temperature for parvalbumin. The component attributed to the solvent exposed peptide bonds undergoes the strongest temperature-dependent shift, whereas the component attributed to the solvent-inaccessible α-helical region of the protein undergoes the weakest temperature-dependent shift (Figure 9).

Some of the IR transitions observed in proteins have no detectable temperature dependence. For transitions involving non-hydrogen bonded groups the IR absorption bands appear to be invariant over a wide range of temperatures. For example, peaks appearing between 2800 and 3000 cm^{-1} that represent CH, CH_2, and CH_3 stretching modes of amino acid residues in the protein did not change in going from room to cryogenic temperature within the level of detection (data not shown). In the case of parvalbumin a shoulder above 1550 cm^{-1} represents the carboxyl stretch for Ca-complexed carboxylates.[28,30] It did not significantly change with temperature, indicating that Ca^{2+} remains bound over the temperature range. These data are pointed out to show that temperature-dependent shifts are not characteristic of all protein associated IR bands, i.e., changing temperature caused no large-scale structural changes.

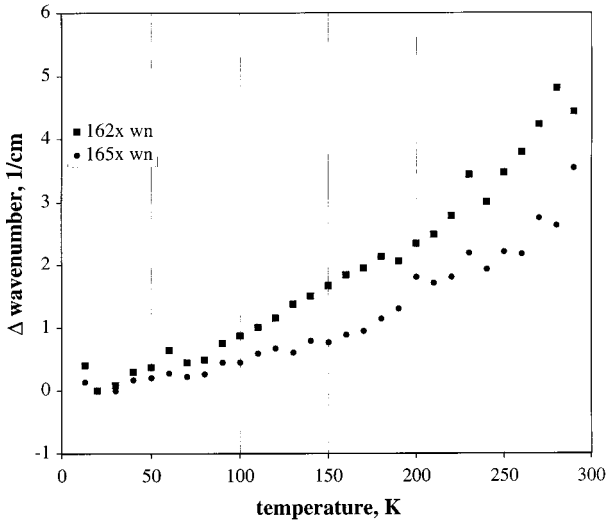

Figure 9. Temperature dependence of the amide I′ frequency of parvalbumin (Figure 8) plotted as the difference from the peak position at 10 K. At 10 K the deconvoluted peak positions were at 1631.6 cm^{-1} (squares) and at 1649.4 cm^{-1} (triangles).

Discussion

In the present study we were interested in whether carbonyl groups in different positions within helical regions could be distinguished by their IR absorption. The isotope effect allowed the spectral separation of particular carbonyl groups. The ^{13}C Ala amide groups in GCN4-P1′ that are exposed to solvent absorb at 1585 cm^{-1}, whereas the amide I′ position for ^{13}C Leu is at 1606 cm^{-1}. The magnitude of difference in the frequency of the amide I′ is sufficiently large so that isotope labeling can allow different positions of the groups within the helix to be monitored.

Further information on the particular bonding types is obtained from the temperature dependence of the IR bands. There is a relatively strong temperature dependence of the amide I′ band of NMA, as well as the low-frequency component of the amide I′ band of α-helical proteins. The amide I′ peak of NMA shifts from 1625 to 1620 cm^{-1} in going from room to cryogenic temperature (Figures 2 and 7). The shift appears to be largest above the solvent glass transition temperature. For the proteins, the amide I′ component attributed to solvent exposed regions is seen just slightly shifted above the amide I′ band of NMA, and it undergoes a similar temperature-dependent shift. The shift for each protein is in the same range as that for NMA. Also similar to NMA, these shifts appear to be strongest above the solvent glass transition temperature (Figure 7a).

In comparison, the component at around 1650 cm^{-1}, typically attributed to α-helical regions of protein, undergoes a significantly weaker temperature-dependent shift. These results suggest that the high-frequency amide I′ component for the proteins can be attributed to buried segments, based on the temperature dependence of the bands. The peak for the buried isotopic labeled Leu in GCN4 likewise did not shift with temperature.

To rationalize the origin of the above effects we consider the possible peptide bond−hydrogen bonds with solvent water molecules by considering the structures of GCN4 and parvalbumin. We found that hydrogen bonds between the proteins and waters of crystallization are observed along the solvent-exposed sides of the helices. Although the helical structures are well-defined, departures from an ideal intrahelical hydrogen bond angle and the potential of the peptide bond carbonyl to form multiple hydrogen bonds still allow for many possible

hydrogen bonds with solvent molecules. This is consistent with a study by Baker and Hubbard,[31] which showed that 53% of the C═O groups in 15 proteins only act as single hydrogen bond acceptors, rather than the expected two. In another study, a relatively large percentage of non-hydrogen bonded C═O groups were attributed to hydrogen bonding to disordered water molecules not identified in X-ray crystal structures.[31]

The amide I transition consists mainly of a CO stretching motion, while amide II consists of a combination of CN stretching and NH in-plane bending.[7] The O (CO)−HO (H_2O) hydrogen bond tends to reduce the force constant of the CO stretch, and thus leads to a shift of the amide I transition to lower frequency. On the other hand, the NH−O (H_2O) hydrogen bond tends to increase the force constant of the NH in-plane bending motion (this motion takes NH−O away from linearity), thus leading to a shift to higher energy of the amide II′ transition. As the temperature is raised, the average hydrogen bond angles increase due to thermal fluctuations. This tends to weaken the hydrogen bonds, reducing the effect of hydrogen bonding on the amide I′ and II′ transitions. Therefore, the amide I′ transition tends to shift to higher frequency and the amide II′ transition tends to shift down with increasing temperature. A similar effect also occurs with the solvent water vibrations, in which case the OH stretching vibrations shift to higher frequency and the bending vibrations shift lower with increasing temperature.[32] On the other hand, the strength of hydrogen bonds existing between peptide linkages in a well-defined α-helix is not expected to exhibit as much of a temperature dependence. This is because the average hydrogen bond angle is not likely to vary as much as in the case where the hydrogen bond is with a small solvent molecule such as water.

Another way to view the temperature effect is to consider coupling of the intramolecular vibrations (CO stretch, NH in-plane bending, water OH stretch, ..., etc, i.e., high-frequency modes) with intermolecular librational motions (i.e., low-frequency modes) that change the hydrogen bonding angle. As the temperature is raised, these low-frequency modes become populated, and can contribute to shifts in the intramolecular vibrational frequencies. In addition, the weakened hydrogen bond network will undergo volume expansion, leading to an additional contribution to the vibrational frequency shifts.[25] Intermolecular librations have characteristic frequencies of tens to hundreds of cm^{-1}. Since kT at room temperature is about 207 cm^{-1}, it follows that changing the temperature will have a marked effect on the hydrogen bond strength, and thus also on the vibrational frequencies. For the case of intrahelix low-frequency motions (such as large scale accordion-like motions of the helix), it is likely that there are fewer of these modes that will change the strength of the intrahelical hydrogen bond, compared to the number of solvent librational modes. In addition, it is likely that the coupling between these large scale motions and the high-frequency amide vibrations is small. For instance, the amplitude of the accordion-like motion of an α-helix (with a frequency of 20−30 cm^{-1}) was calculated to be 0.2 A for an 11 amino acid helix in insulin.[33] If such a displacement is distributed over several helical turns, then the average change in hydrogen-bond length between the peptide linkages is small. Furthermore, as mentioned above, the average H-bond angle is unlikely to change as much during an accordion-like motion compared to a solvent librational mode. Using the amplitude of vibration from ref 33, we calculate that the intrahelical hydrogen bond angle will only change by about ±0.5 degrees. Thus, since the individual hydrogen bond strengths are not expected to change substantially during the course of the large-scale motion, these low-frequency intrahelical vibrations are not likely to affect the amide vibrations significantly. Therefore, the amide bands of a buried helix are likely to exhibit weaker temperature dependence than those of a solvent-exposed helix.

The above explanations are consistent with a theoretical study by Reisdorf and Krimm,[14] which suggested that unusually low-frequency amide I′ transitions in helical proteins such as tropomyosin, calmodulin, and troponin C[34] could be attributed to the additional hydrogen bonding of solvent accessible backbone CO groups to solvent water molecules. In this study, it was shown that the profile of the 3-component amide I′ band of tropomyosin could be reproduced by using an empirical force field to calculate the vibrational eigenmodes of a coiled-coil, with small random variations in the dihedral angles ϕ and ψ. However, hydrogen bonding between the helix CO groups to water was invoked to explain the position of the low-energy (1630 cm^{-1}) component of the band.

The types of interactions discussed above will have an effect on the IR spectra, and the use of isotopically labeled peptides may help to elucidate origins of spectral shifts. In an ideal helix, it has been pointed out that coupling of the CO stretches along the helix can result in a vibrationally allowed exciton that is shifted in energy relative to that of an individual CO stretch.[7,35−37] Disruption of the periodicity by bending the helix or introducing random variations in the structure may cause the excitons to become localized in "islands", similar to those which occur with optical Frenkel excitons.[24] When one side of the helix is exposed to a hydrogen-bonding solvent, a modulation in the CO stretching frequency along the helix would be introduced, yielding a split exciton band. It is also possible that a vibrational exciton localized in a solvent exposed region of the helix would be more sensitive to temperature, simply because the absorption band is arising from several carbonyls, each involved in H-bonding with the solvent. Excitons localized in regions not exposed to solvent might then exhibit a weaker temperature dependence.

Finally, we mention other possibilities for the spectral differences between solvent exposed and buried amide groups. Surface carbonyl groups in helices are also known to have small geometric differences relative to the buried amides[38,3938,39] which could possibly account for the shifts we observe in the amide I′ bands. However, this appears less likely because we observe the same temperature-dependent shifts in *N*-methylacetamide. Another possibility is that low-frequency modes could combine and come into resonance with the amide I′ band. If these modes involve atoms taking part in hydrogen bonding they could contribute to the temperature-dependent shifts. However, the probability of this leading to the observed trends is quite low, and even if this did occur, we feel that it is unlikely to account for the observed trends.

Hydrogen bonding between water and the surface carbonyls in helices has a structural consequence on the helix, and therefore spectroscopic evidence, such as shown in this work,

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is of interest. The intrahelical hydrogen bond tends to be longer (3.1 Å versus 2.9 Å for buried amides) and less linear (148° versus 157° in buried amides).[31,39] The significance of H-bonding between solvent and water exposed amide groups is emphasized by recent work,[40] which demonstrated that water−amide interactions stabilize helices by an enthalpic factor. Luo and Baldwin showed that Ala is a strong helix-forming amino acid because it allows such interactions, while the more sterically encumbered hydrophobic amino acids are not as good helix formers because they block access of the carbonyls to water. The finding that the α-helical regions show differences in spectral properties based upon water accessibility may be useful to monitor protein−protein or protein−membrane interactions, i.e., situations where exposure to water changes. Water-accessibility of one face for surface helices in general and GCN4-P1′ in particular[20] can account for the periodic shifts in the amide NH chemical shifts. IR results can complement such NMR studies, where IR gives information on the instantaneous profile of H-bonding, and NMR includes a factor for kinetic effects.

In summary, the amide group exposed to solvent, represented by NMA, shows a relatively strong dependence on temperature, and the shifts are consistent with increased hydrogen bonding at low temperature. Isotopic labeling of GCN4-P1′ definitively showed that the position and temperature dependence of peaks differ for buried and solvent exposed residues. Analysis of the temperature dependence of IR frequency modes in the proteins indicates that one population of amide bonds in proteins has a similar temperature dependence to model compounds in the aqueous environment. The data attest that the temperature dependencies of the amide I′ frequencies follow the glass transition of the solvent. Amide bonds in α-helical regions that are excluded from water exhibit weaker temperature dependence.

Experimental Section

Sample Preparation. The synthetic derivatives GCN4-P1′ were prepared as previously described.[22,41−43] Parvalbumin was prepared from codfish, and then lyophilized.[44] NMA, D_2O, perdeuterated glycerol, and other chemicals were obtained from Sigma/Aldrich Chemical Co. (St. Louis MO). Water was deionized and then glass distilled.

The samples were prepared as follows: the lyophilized protein samples were dissolved in pD 7.0 phosphate buffered D_2O. The samples were allowed to incubate $>\sim$24 h for parvalbumin. For the peptides, after standard peptide synthesis, purification, and lyophilization, the sample was dissolved in 1 M HCl solution and lyophilized to replace TFA with HCl. The process was repeated, i.e., redissolved in 1 M HCl and lyophilized, to ensure complete removal of TFA. Then 10 mg of peptide was dissolved in 0.5 mL of phosphate buffer (5 mM phosphate, pH (pD) about 7.5) in D_2O and deuterated phosphate buffer and allowed to sit at room temperature for 3 days. This incubation length ensured D to H exchange of most of the amide bonds. To carry out low-temperature measurements, a cryosolvent must be added to the D_2O. Prior to measurement all samples were diluted in perdeuterated glycerol to give a 50% v/v solution.

Instrumentation and Data Analysis. Infrared (IR) spectra were obtained with a Bruker IFS 66 Fourier transform IR instrument (Bruker Inc., Brookline MA) as previously described.[45] The sample compartment was purged with nitrogen to reduce the contribution from water vapor. The light levels were monitored using an HgCdTe (MCT) detector. The spectral resolution was 2 cm^{-1}. The spectra were smoothed using a 9 Savitzky-Golay smoothing algorithm. The sample holder was obtained from Graseby Specac (Smyrna GA 30082). A 15 mm spacer was used between two CaF_2 windows in the transmission cell. The temperature was regulated using an APD closed cycle Helitran cryostat (Advanced Research Systems, Allentown, PA). The cryostat sample chamber was filled with He gas at atmospheric pressure, which aids in the transfer of heat from the sample. The outer cryostat windows were made of CaF_2. The inner cryostat windows, which experience the temperature gradient, were 2 mm thick and were made of ZnSe (Janos Technology, Inc. Townsend VT). A special holder for these windows was constructed to minimize strain on the windows due to contraction at low temperature (Research Instrumentation Shop, University of Pennsylvania School of Medicine, Philadelphia, PA 19104). The temperature was measured with a silicon diode near the sample and the temperature was controlled using a Model 9650 temperature controller (Scientific Instruments, Inc., W. Palm Beach FL 33407). Cryogenic temperature profiles were carried out from high to low temperature. The temperature was measured every 10 deg; the time for equilibration for each temperature took 5 to 10 min. The spectra were analyzed for their components using the PeakFit software package (Jandel Scientific Software, CA).

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