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Impact of Four ^{13}C-Proline Isotope Labels on the Infrared Spectra of Ribonuclease T1

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Abstract: Ribonuclease T1 was biosynthesized, with all four prolines ^{13}C-labeled in the peptide C═O bond, using a proline auxotrophic yeast strain of *Saccharomyces cerevisiae*. The ^{13}C- and ^{12}C-proline isotopomers of ribonuclease T1 were investigated by infrared spectroscopy in the thermally unfolded and natively folded state at 80 and 20 °C, respectively. In the thermally unfolded state, both proteins established almost indistinguishable spectral features in the secondary structure sensitive amide I region. In contrast, the spectra measured at 20 °C revealed substantial qualitative and quantitative differences, though parallel analysis by circular dichroism suggested identical native folds for both isotopomers. Major spectral differences in the infrared spectra were detected at 1626 and 1679 cm^{-1}, which are diagnostic marker bands for antiparallel β-sheets in ribonuclease T1 and at 1645 cm^{-1}, a region that is characteristic for the infrared absorption of irregular structures. Starting with the known three-dimensional structure of ribonuclease T1, the observed effects of the isotope labeling are discussed on the basis of transition dipole coupling between the ^{12}C═O and ^{13}C═O groups. The experimental results were confirmed by transition dipole coupling calculations of the amide I manifold of the labeled and unlabeled variant.

Introduction

The amide I bands in the IR spectra of proteins between 1600 and 1700 cm^{-1}, energetically mainly C═O stretching vibration, are established indicators of the polypeptide backbone geometry and have frequently been used to scrutinize protein secondary structures.[1−3] However, the IR technique, unlike nuclear magnetic resonance, cannot resolve the conformation of individual peptide residues at specific sites. A way to overcome this limitation is to substitute single ^{12}C═O groups with a ^{13}C═O group in a given polypeptide chain. This method is known as isotope edited infrared spectroscopy.

Several authors have shown that the impact of ^{13}C-labeled amino acids on the amide I region strongly depends on their conformational surrounding. In isotopically labeled α-helical structures where transition dipole coupling (TDC) between the C═O oscillators does not cause an observable band splitting, the amide I vibrational frequency decreases by 35 to 45 cm^{-1} due to the increased mass, as demonstrated for a synthetic 28-amino acid fragment of the membrane protein phospholambane[4] and a series of alanine-rich helical peptides.[5] A downward shift of the amide I band of 40−45 cm^{-1} was also observed after uniformly biosynthetic labeling of proteins with ^{13}C[6,7] or by site-specific ^{13}C-labeling of the α-helical protein bacteriorodopsin.[8] In contrast, ^{13}C-labeling of amino acids that are part of the intermolecular antiparallel β-sheet of a nine-amino acid peptide, changed the spectral patterns of the amide I contour dramatically.[9] These observations could not be explained on the basis of pure mass-related variations of the amide I carbonyl oscillator. Recently it was shown that additional to the increased mass, through-bond interactions and TDC also have to be taken into account to bring theoretical considerations in accordance with experimental observations.[10]

In the present study, we describe the impact of four carbonyl ^{13}C labels on the FT-IR spectrum of the well-characterized 104-

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amino acid α+β-protein ribonuclease T1 (RNase T1) in the folded and unfolded state. We substituted in RNase T1 the ^{12}C carbonyl carbons of the four prolines with their heavier isotope ^{13}C using a proline auxotrophic yeast strain of *Saccharomyces cerivisiae* as the expression system.

To interpret the impact of the isotope labels on the IR spectra of RNase T1, we have modeled the difference spectra between the native and the ^{13}C -substituted protein by TDC computations in the amide I spectral manifold.

Material and Methods

Materials. Glucose, galactose, and TRIS were purchased from Merck (Darmstadt, Germany), and NaCl, EDTA, $(NH_4)HCO_3$, ^{12}C-L-proline, and RNase T1 standard from *Aspergillus oriyzae* were from Sigma (Taufkirchen, Germany). The yeast nitrogen base without amino acids was purchased from Difco (Detroit, MI), the HPLC eluents were from J. T. Baker (Deventer, The Netherlands), and the L-proline (1-^{13}C, 99%) was from Promochem (Wesel, Germany).

Protein Expression and Purification. The unlabeled and ^{13}C-labeled RNase T1 were synthesized using a proline auxtrophic *S. cerevisiae* strain as the expression system. The proline and uracil auxotrophic *S. cerevisiae* strain DT1103 was a generous gift from Marjorie C. Brandriss (New Jersey Medical School). The vector pDP(α)T1 was provided by U. Hahn (Leipzig, Germany). This vector complements the uracil auxotrophy and carries the gene for the galactose-regulated expression of RNaseT1 into the medium. The transformation of the yeast cells with pDP(α)T1 was performed by applying the LiAc method of Schiestl and Gietz.[11]

S. cerevisiae DT1103 carrying the vector pDP(α)T1 were cultivated at 30 °C on Petri dishes containing 2% glucose, 0.1% proline, 0.67% yeast nitrogen base (YNB) without amino acids, and 1,6% agarose. An overnight liquid culture of *S. cerevisiae* containing 2% glucose, 0.67% YNB, and 0.1% proline was used to inoculate a culture containing 0.5% glucose, 1.25% galactose, 0.67% YNB, and 0.005% proline. Subsequently, the culture was incubated in a shaker for 4 days. After incubation, the medium was tested for enzyme activity by an assay described elsewhere.[12]

The culture was centrifuged (8000*g*, 20 min, 4 °C), the supernatants were combined, and the pellets were discarded. Afterward, the supernatants were sterile filtered, dialyzed against 25 mM Tris-HCl/5 mM EDTA, pH 7 (TE25) (Spectrapor Membrane 1 MWCO: 6−8000) and loaded at a flow rate of 5 mL/min on a DEAE Fractogel EMD 650 (s) (Merck) column (3 × 16 cm) preequilibrated with TE25. After loading, the column was washed with five column volumes of TE25 containing 150 mM NaCl. The RNase T1 was then eluted isocratically at a flow rate of 2 mL/min. with TE25 containing 350 mM NaCl. Finally, the fraction was desalted by loading on a Sephadex G25 fine (Pharmacia) column (5 × 35 cm) with 0.1 mol/L $(NH_4)HCO_3$ buffer, pH 7.9, as eluent and lyophilized.

The lyophilized protein was dissolved in a small volume of 0.1 mol/L $(NH_4)HCO_3$ buffer, pH 7.9, passed through a 0.45-μm filter and loaded on a Nucleogel RP 100-8 (Macherey-Nagel) column connected to a Hewlett-Packard (HP) 1050. Since former purification attempts showed that TFA was difficult to eliminate from the RNase T1 and caused an interference in the amide I region by the carboxyl band at 1672 cm^{-1}, we used an elution system of 0.1 mol/L $(NH_4)HCO_3$, pH 7.9 (eluent A) and 60% acetonitrile (eluent B). Hence, the RNase T1 was eluted with a gradient from 20 to 80% eluent B at a flow rate of 1.5 mL/min. The elution of RNase T1 was monitored at 278 nm at 56% *e*luent B. Finally, the protein was lyophilized for further measurements. The purification by reversed-phase (RP) chromatography has proven to produce RNase T1 of excellent purity. We think that the use of a RP column is also advantageous in the purification process of RNase T1 from *Escherichia coli*.[13,14]

The purity of the protein was determined by RP-HPLC. The total amount of protein was evaluated by integration of the HPLC peaks using RNase T1 standard for calibration. The enzymatic activity of the labeled and unlabeled proteins was determined using an assay described elsewhere.[15] This purification process yielded ∼200 μg of RNase T1 from 1 L of cell culture. The specific activities (ΔA_{257}/(min mg)) of the unlabeled and ^{13}C-labeled variants were ∼115.

CD Spectroscopy. Spectra of ^{12}C- and ^{13}C-labeled RNase T1 were recorded on a Jasco J-720 CD spectrometer. The spectra were acquired at room temperature from 260 to 195 nm using a cell with a path length of 1 mm. The samples were dissolved in 5 mM sodium cacodylate buffer, pH 7.0, at a concentration of ∼0.15 mg/mL.

FT-IR Spectroscopy. For infrared measurements, samples of the unlabeled and labeled RNase T1 were dissolved at a concentration of 10 mg/mL in 100 mM sodium cacodylate/D_2O buffer at pH* 7.1 (where pH* is the pH electrode reading without correction for the isotope effect). All infrared spectra were recorded using a Bruker IFS-66 FT-IR spectrometer equipped with deuterated triglyceride sulfate detector and a shuttle system. To eliminate spectral contributions due to atmospheric water vapor, the instrument was continuously purged with dry air. The samples (∼10 μL) were placed into a thermostated CaF_2 cell with a fixed path length of ∼50 μm. For each spectrum, 128 background and sample scans were coadded and averaged. Fourier transformation was done using a Happ-Genzel apodization function, a zero filling factor of 4, and a nominal resolution of 4 wavenumbers. All protein spectra were corrected for buffer and water vapor as described previously.[16] Band narrowing by Fourier self-deconvolution (FSD) was performed applying a Lorentzian line shape function with a band with of 2.7 and a resolution enhancement of 2.2.

TDC Calculations. For a peptide or protein of *k* individual residues, we denote the amide I vibrational frequency and dipole transition moment of the *i*th residue by ν_i and μ_i, respectively.[17] In the dipole−dipole approximations, these transition moments interact, or couple. We define the interaction matrix of all dipoles as

$$\mathbf{V}_{ij} = \frac{\mu_i \cdot \mu_j}{|\mathbf{T}_{ij}|^3} - 3\frac{(\mu_i \cdot \mathbf{T}_{ij})(\mu_j \cdot \mathbf{T}_{ij})}{|\mathbf{T}_{ij}|^5} \quad \text{for} \quad i \neq j \tag{1a}$$

and

$$\mathbf{V}_{ij} = \nu_i \quad \text{for} \quad i \neq j \tag{1b}$$

Here, $\mathbf{T}_{ij}$ is a vector connecting the centers of mass of oscillators *i* and *j*. As indicated by eq 1b, the diagonal elements of this matrix are the unperturbed transition frequencies (in wavenumber) of the individual amide I stretching modes, which need not be degenerate. Of course, the coupling energy of the transition dipoles needs to be converted to wavenumber units before diagonalization of the **V** matrix. This diagonalization yields *k* "exciton" frequencies for the interacting dipoles. Thus, the resulting transitions can no longer be described as individual amide I transitions, but as linear combinations of all interacting dipoles.

The contribution of each individual transition to the *k*th exciton transition is given by the eigenvector of **V**. Furthermore, the dipole

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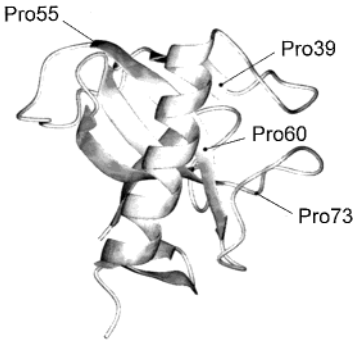

Figure 1. Crystal structure of RNase T1 generated by Molmol 2.5.1 applying the atomic coordinates of the file 9RNT obtained from the Brookhaven Protein Database. The four prolines present in RNase T1 are indicated.

strengths D_k of the kth transition is given

$$D_k = \sum_{i=1}^{N} \cdot \sum_{j=1}^{N} c_{ik} c_{jk} (\mu_i \cdot \mu_j) \qquad (2)$$

For the calculations reported below, all transition dipole moments, μ_i, were taken to be equal, with a value of 950 L/(mol cm). Calculated dipole strengths (in esu^2 cm^2) were converted to units of L/(mol cm) by the approximation

$$D \approx 9.2 \times 10^{-39} \pi \epsilon_{max} w/\nu_0 \qquad (3)$$

for Lorentzian band shapes. Here, w is the full width at half-height of the band. The calculations for the wild-type species were carried out with the following input parameters: $\nu_0 = 1660$ cm^{-1} for all secondary amide I functions and $\nu_0 = 1637$ cm^{-1} for all tertiary (prolyl) amide I functions at residues 54, 38, 59, and 72. Furthermore, bandwidths of $w = 12$ cm^{-1} and extinction coefficients $\epsilon_{max} = 950$ L/(mol cm) were assumed. For the ^{13}C-substituted protein, $\nu_o = 1623$ cm^{-1} was used at residues 55, 39, 60, and 73.

Results and Discussion

Figure 1 displays the crystal structure of RNase T1 with the four ^{13}C-labeled prolines highlighted.[18] The three-dimensional structure of the RNase T1 is characterized by an α-helix of 4.5 coils, an extended antiparallel β-sheet consisting of three long and two short β-strands, a short two-stranded antiparallel β-sheet, four wide loops, several types of turns, and two disulfide bonds connecting the C- and N-terminal region.[18] The prolines at positions 39, 55, and 60 are part of the extended antiparallel β-sheet structure, whereas the proline at position 73 is located in a wide loop.

Figure 2 shows the CD spectra of the unlabeled and ^{13}C-proline-labeled RNase T1 at 20 °C in its native conformation. Both spectra are nearly identical, indicating that the substitution of the four proline residues in RNase T1 by ^{13}C-proline has no impact on the secondary structure of the protein.

Figure 3a shows the FT-IR spectra of the same proteins fully H/D exchanged in D_2O buffer using the sharp tyrosine side-chain band at 1515 cm^{-1} as an internal standard for normalization. In contrast to the CD spectra, the IR spectra of the two isotopomers exhibit a number of distinct spectral differences in the amide I contour. The major amide I features at 1643 and

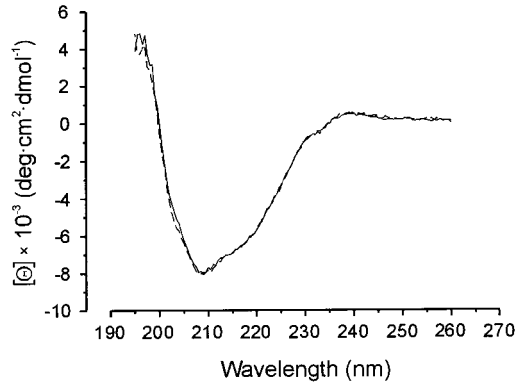

Figure 2. CD spectra of the proline ^{13}C-labeled RNase T1 (solid line) und the unlabeled RNase T1 (dashed line), both measured at 20 °C.

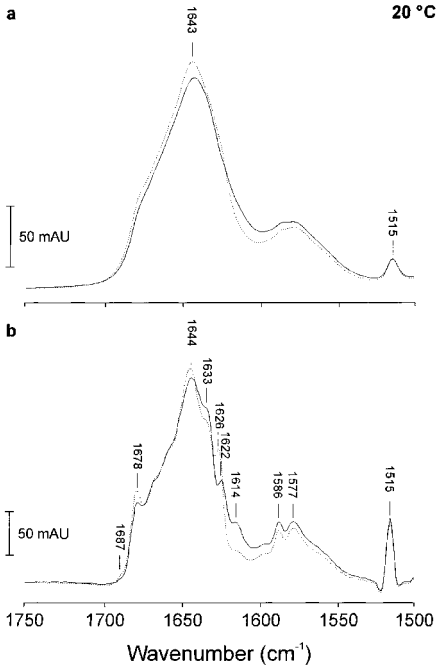

Figure 3. Infrared spectra of the unlabeled (dashed lines) and ^{13}C-labeled (solid lines) RNase T1 at 20 °C. (a) Overlaid absorbance spectra as measured using the tyrosine band at 1515 cm^{-1} as an internal intensity standard for spectra normalization. (b) Infrared spectra after band narrowing by Fourier self-deconvolution.

1678 cm^{-1} appear more pronounced for the unlabeled protein, whereas the isotope-labeled enzyme shows higher intensity at 1600 cm^{-1}.

Figure 3b displays the FSD spectra of the unlabeled and ^{13}C-labeled RNase T1. The spectral fine structure of the unlabeled protein is the same as already described in the literature.[16,19] The bands at 1626 and 1678 cm^{-1} have been described to be diagnostic for antiparallel β-sheets in RNase T1. This split amide I mode arises from TDC between the C═O oscillators in the β-sheet core.[20−22] The component band at 1644 cm^{-1} has been tentatively assigned to overlapping bands of irregular structures, turns, and loops and presumably the α-helix.[16,19] Spectral contributions between 1610 and 1500 cm^{-1} of fully H/D-exchanged proteins are due to amino acid side-chain vibra-

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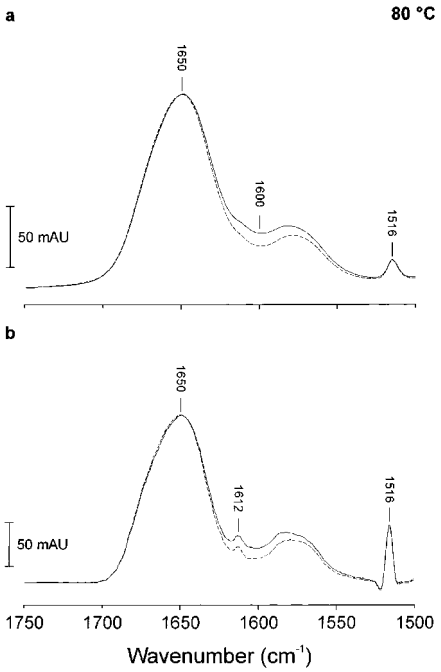

Figure 4. Infrared spectra of the unlabeled (dashed lines) and ^{13}C-labeled (solid lines) RNase T1 at 80 °C. (a) Overlaid absorbance spectra as measured using the tyrosine band at 1515 cm^{-1} for normalization. (b) Infrared spectra after band narrowing by Fourier self-deconvolution.

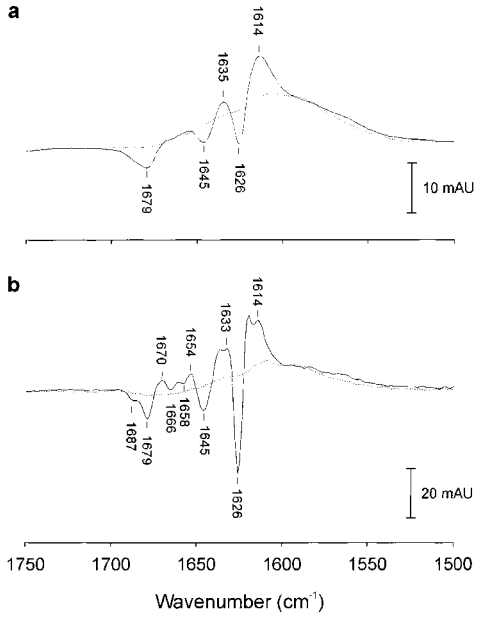

Figure 5. Difference spectra at 20 (solid lines) and 80 °C (dashed lines) between the ^{13}C-labeled and unlabeled RNase T1. (a) Difference spectra of the absorbance spectra. (b) Difference spectra of the resolution enhanced spectra.

tions.[16,23] Hence, the bands at 1586 and 1577 cm^{-1} arise from asymmetric carboxylate stretching vibrations of the amino acid side-chain groups of aspartate and glutamate, respectively. The band at 1515 cm^{-1}, which is assigned to the aromatic ring stretching vibration of the tyrosine side chain residues of the protein, is generally considered as a sensitive marker band for tertiary structure formation.[19,24] The identical frequency position of the tyrosine band for both proteins lends further support for the assumption that the three-dimensional fold of the isotopomers is the same.

The FSD spectra of Figure 3b exhibit unexpectedly prominent differences between the labeled and unlabeled RNase T1. The main differences clearly occur for the antiparallel β-sheet marker bands. The high-frequency component band of the labeled protein at 1678 cm^{-1} shows significantly reduced intensity. The ^{13}C-labeled protein, in contrast, has higher band intensities than the unlabeled enzyme at 1633 and 1614 cm^{-1}.

The corresponding FT-IR spectra of the thermally unfolded proteins are given in Figure 4. Both, the original and the FSD spectra (panels a and b of Figure 4, respectively) show, as expected, only a broad nearly featureless amide I band contour around 1650 cm^{-1}. As already described for peptides, spectral differences specifically arising from ^{13}C-labeling are not well resolved in the thermally unfolded state when protein secondary structure is destroyed.[25] Under these conditions, all defined hydrogen bonds can be considered to be broken down and TDC is impossible, since its effectiveness is very sensitive to the distances and orientations of the related dipoles.

The difference spectra of Figure 5 reveal the spectral alterations due to the labeled prolines in more detail. These spectra were generated by subtracting the original (Figure 5a) or the FSD spectra (Figure 5b) of ^{12}C RNase T1 from the corresponding spectra of the ^{13}C-labeled protein at 20 and 80 °C, respectively, using the tyrosine band at 1515 cm^{-1} as an internal standard. The difference spectra obtained at 20 °C (Figure 5a and b, solid lines) demonstrate nicely that major intensity changes are observed for the antiparallel β-sheet marker bands at 1626 and 1679 cm^{-1} and for the band at 1645 cm^{-1}. Interestingly, the high-frequency β-band at 1679 cm^{-1} shows a negative band component that is fairly broad. The corresponding FSD difference spectrum of Figure 5b (solid lines) reveals two high-frequency components at 1687 and 1679 cm^{-1} and a small positive feature at 1670 cm^{-1}. In addition, the difference spectrum shows increased intensity at 1633 and at 1614 cm^{-1}.

The magnitude of the shift (toward lower wavenumbers) for an isolated ^{12}C═O stretching frequency after substitution by a ^{13}C isotope is expected to be in the order of 35−40 cm^{-1}.[26] Figure 5 impressively demonstrates that the spectral differences between the labeled and unlabeled protein can only be detected in the folded state. These spectral differences are substantially larger than expected from four labeled exchanged C═O oscillators. In particular the β-sheet marker bands at 1626 and 1679 cm^{-1} experience a pronounced intensity loss. There are two related factors that may account for this observation. Kubelka and Keiderling reported that phase effects of coupled oscillations can account for very large differences.[27] Furthermore, in the case of substitution at the proline carbonyl group, a further coupling effect may take place. This effect appears to be due to coupling of the tertiary amide vibration next to the N-terminus of proline with the ^{13}C-substituted carbonyl group at the C-terminal of proline. Both these vibrations are significantly lower than the regular amide I vibration, at 1637 and 1623 cm^{-1}. The proximity in energy of these vibrations may enhance their

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interaction and leads to enhanced coupling with larger isotopic effects than expected.

As mentioned above, the splitting of the β-sheet band into a low- and high-frequency component results from TDC. We therefore state that the observed lower intensity of the β-sheet marker bands at 1626 and 1679 cm^{-1} is a direct consequence of reduced TDC, brought about by the "uncoupling effect" of one or more of the ^{13}C-labeled amide I carbonyls within the β-sheet. Since TDC depends on the vibrational frequencies of the coupling dipoles, at least one of the substituted prolines with uncoupled $^{13}C{=}O$ dipoles must be responsible for the reduced TDC and can, therefore, undoubtedly be localized in the β-sheet region. In fact, the available crystal structure of RNase T1 (see Figure 1) indicates that three of the prolines are located close to or even within the extended β-sheet.[18] The prolines at positions 55 and 60 are part of the extended β-sheet, while the proline at position 39 is rather located at the end of this β-sheet strand, close to a π- and β-turn system. The proline at position 73 is localized within a loop structure.

In addition to the reduced TDC caused by the uncoupling effect of ^{13}C-labeling, the overall coupling of the "residual" ^{12}C-amide groups within the β-sheet is expected to be reduced, resulting in a shift of the ^{12}C-amide I β-sheet absorption band to higher frequencies.[21,22] The positive difference band at 1633 cm^{-1} (Figure 5b) is possibly due to this effect. The extent of this shift strongly depends on the positions of the corresponding dipoles in the β-sheet, as was recently shown for an amyloid-forming peptide.[9]

The high-frequency band component at 1679 cm^{-1} is of particular interest. In the FSD difference spectrum (Figure 5b), this band splits up in two components with a band of medium intensity at 1679 and a minor component at 1687 cm^{-1}, suggesting that these features stand for two different structure elements.

Besides the high-frequency β-sheet band component, it is also known that turn structures may give rise to absorptions in the spectral region around 1680 cm^{-1} and also between 1640 ad 1660 cm^{-1} [1]. Krimm showed that the absorbance frequencies and relative intensities of turn structures are also influenced by TDC, depending on the dihedral angles defining the particular turns.[1] Consequently, ^{13}C-labeling of amide carbonyls within or close to turn structures will also influence the turn-specific absorptions in the amide I region via TDC. We therefore propose that the ^{13}C-labeled proline in position 39 interferes with the TDC system of the π-turn produced by amino acids 33−38 and the β-turn comprising the amino acids at positions 34 and 37, leading to distinct alterations in the spectral region around 1687 cm^{-1}. Furthermore, we propose that the negative difference band at 1645 cm^{-1} is also due to the "uncoupling effect" of the labeled proline at position 39 since turn structures might give rise to absorptions in this spectral region. Computational results validating these assignments will be presented at the end of the Results and Discussion section.

An interesting feature in the difference spectrum of Figure 5 is the positive band intensity at 1614 cm^{-1}. Similar features were observed by several authors in a variety of ^{13}C-labeled peptides and proteins. It was shown for the membrane protein phospholamban that a band in this region appears from an isotope-induced downshift of 44 cm^{-1} of the α-helix band at 1658 cm^{-1}, typical of membrane proteins.[4] In β-sheet-forming peptides, absorption bands between 1600 and 1620 cm^{-1} were attributed either to coupling between the $^{13}C{=}O$-labeled dipoles [9] or as the result of combined ^{13}C group and ^{12}C group motions by through bond coupling.[10] Since the α-helix of RNase T1 has not been labeled, through bond coupling between the labeled und unlabeled C=O oscillators as proposed by Brauner et al.[10] seems one plausible explanation. Recently, Kubelka and Keiderling argued on the basis of ab initio calculations that the enhancement of this band seems to result from a single mode that is predominantly due to ^{13}C and whose largest amplitudes are on the center strand or strands of the multistranded structure of their model polypeptide.[27] In the case of RNase T1, Pro60 might be the major contributor to this band.

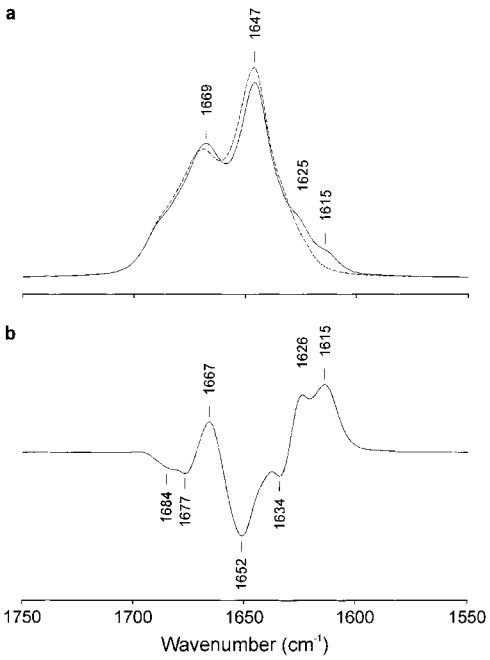

Figure 6. Calculated absorbance spectra of the labeled and unlabeled RNase T1 (a) and the corresponding difference spectrum (b). The intensities of the difference spectrum were magnified by a factor of 4.

To interpret the impact of the isotope labels on the IR spectra of RNase T1, we have modeled the difference spectra between the native and the ^{13}C-substituted protein by TDC computations in the amide I spectral manifold. The size of this protein make ab initio calculations of the infrared spectral features impractical at this time, although efforts in this direction were reported recently for smaller proteins and protein segments.[27]

TDC calculations represent a somewhat simplistic approach to computing infrared spectral features. However, this approach was used quite successfully in the computations of infrared (vibrational) circular dichroism (VCD) spectra of peptides and nucleic acid fragments.[28] In short, this method, also known as the "coupled oscillator" model, is based on the concept that the bandwidth and splitting in the amide I spectral manifold is due to dipolar coupling of the amide I transitions of each individual amide group. The known three-dimensional crystal structure of the RNase T1 served as the source for the coordinates of the C=O groups involved in the calculations. In crystals, proteins are highly solvated and therefore may resemble the structure in solution. Asides from crystal structures, the coordinates obtained

(28) Xiang, T.; Goss, D. J.; Diem, M. *Biophys. J.* **1993**, *65*, 14872−14877.

by NMR analysis or molecular dynamics (MD) simulations might be used for the calculations.[29]

Figure 6 shows the absorbance spectra of the labeled and unlabeled RNase T1 variants (a) and the corresponding difference spectrum (b) generated by TDC calculations. The computations were carried out as described in the Material and Methods section. In contrast to ab initio calculations, which require a few weeks of computation times on a supercomputer, TDC modeling of the protein amide I manifold is trivial and can be carried out in a few seconds on a PC. Although all other contributions to the amide I contour, aside from TDC, are ignored in this approach, there is a high similarity to the corresponding experimental spectra in Figure 5. Particularly remarkable is the agreement of the low- and high-frequency component bands around 1615 and 1680 cm^{-1}, respectively. In analogy to the experimental spectra, there is a shoulder in the computed spectra at 1684 cm^{-1} (observed at 1687 cm^{-1}) whereas the low-frequency β-sheet band at 1626 cm^{-1} in the experimental spectra seems to be shifted to 1634 cm^{-1} in the modeled spectrum. The positive component in the calculated difference spectrum at 1667 cm^{-1} is more pronounced than in the corresponding experimental difference spectrum. The major results from these calculations give a more detailed insight into the impact of ^{13}C labels on the vibrational spectra.

To verify the assignments of the observed proline residues, we simulated the IR absorption spectra of all four (single proline) ^{13}C-substituted isotopomers. These calculations demonstrated that all single ^{13}C substitutions produce spectral changes that span the entire amide I manifold. The amplitudes of the difference spectra (unsubstituted − monosubstituted) was smallest for Pro73 ($\Delta\epsilon/\epsilon = 2 \times 10^3/3.5 \times 10^4$), which resides in a wide loop region, and largest for Pro55 and Pro60 ($4.5 \times 10^3/3.5 \times 10^4$ and $3.6 \times 10^3/3.5 \times 10^4$, respectively). These residues are found in extended β-sheet regions. However, most significant was the fact that the simulated spectra for both Pro39 and Pro55 substitution caused a significant intensity decrease at 1675 and 1674 cm^{-1}, respectively. This observation confirms the assignment presented above, that the high wavenumber shoulder of the amide I manifold is due to Pro39 and Pro35, which represent the two *cis*-proline residues in native RNase T1. Furthermore, isotopic labeling at these residues also caused significant intensity decrease between 1635 and 1645 cm^{-1}.

(29) Torii, J.; Tasumi, M. *J. Chem. Phys.* **1991**, *96*, 3379−3387.

Conclusion

The amide I region "encodes" detailed information about structures and dynamics of proteins on the peptide group level, which typically cannot be extracted easily. In the present study, we demonstrate that a combined approach of isotope edited FT-IR spectroscopy and spectral simulations on the basis of TDC calculations has the potential to reveal some of the structural and dynamic information contained in the amide I manifold. Although TDC modeling is the most simplistic of the spectral simulation methods, the calculated spectra are in reasonable agreement with the observed amide I bands of the isotopically labeled and unlabeled RNase T1 and served as a standard for the verification of our assignments. The crystal structure of the protein served as the source for the input coordinates of the C═O groups needed for the calculations.

The four labeled prolines in RNase T1 are of specific interest because three of them (at positions 39, 55, and 60) are located in β-sheet structures that give rise to characteristically split absorbance IR bands. It is known that the formation of intermolecular β-sheet structures, in particular, proteins, is the cause of many diseases such as Alzheimer and transmissible spongiform encephalopathies. RNase T1 enriched with isotope labels in this structural element was therefore chosen as a model system for obtaining a deeper insight into β-sheet structures by IR spectroscopy. We think that the method presented in this study might also provide a valuable tool for investigating specific conformational changes in sophisticatedly labeled biomedically relevant proteins especially in combination with stopped-flow and time-resolved IR detection techniques. First promising efforts leading in this direction are currently underway: Time-resolved temperature jump experiments on labeled and unlabeled RNase T1 variants enable the direct detection of the Pro39 and Pro55 trans → cis isomerization reaction in the refolding course of this protein.

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