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Investigation of protein–protein interactions by isotope-edited Fourier transformed infrared spectroscopy

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Abstract. Recent advance in FTIR spectroscopy has shown the usefulness of ^{13}C uniform isotope labeling in proteins to study protein–protein interactions. ^{13}C uniform isotope labeling can significantly resolve the spectral overlap in the amide I/I′ region in the spectra of protein–protein complexes, and therefore allows more accurate determination of secondary structures of individual protein component in the complex than does the conventional FTIR spectroscopy. Only a limited number of biophysical techniques can be used effectively to obtain structural information of large protein–protein complex in solution. Though X-ray crystallography and NMR have been used to provide structural information of proteins at atomic resolution, they are limited either by the ability of protein to crystallize or the large molecular weight of protein. Vibrational spectroscopy, including FTIR and Raman spectroscopies, has been extensively employed to investigate secondary structures and conformational dynamics of protein–protein complexes. However, significant spectral overlap in the amide I/I′ region in the spectra of protein–protein complexes often hinders the utilization of vibrational spectroscopy in the study of protein–protein complex. In this review, we shall discuss our recent work involving the application of isotope labeled FTIR to the investigation of protein–protein complexes such as cytokine–receptor complexes. One of the examples involves G-CSF/receptor complex. To determine unambiguously the conformations of G-CSF and the receptor in the complex, we have prepared uniformly $^{13}C/^{15}N$ isotope labeled G-CSF to resolve its amide I′ band from that of its receptor in the IR spectrum of the complex. Conformational changes and structural stability of individual protein subunit in G-CSF/receptor complex have then been investigated by using FTIR spectroscopy (Li et al., *Biochemistry* **29** (1997), 8849–8859). Another example involves BDNF/trkB complex in which $^{13}C/^{15}N$ uniformly labeled BDNF is complexed with its receptor trkB (Li et al., *Biopolymers* **67**(1) (2002), 10–19). Interactions of $^{13}C/^{15}N$ uniformly labeled brain-derived neurotrophic factor (BDNF) with the extracellular domain of its receptor, trkB, have been investigated by employing FTIR spectroscopy. Conformational changes and structural stability and dynamics of BDNF/trkB complex have been determined unambiguously by FTIR spectroscopy, since amide I/I′ bands of $^{13}C/^{15}N$ labeled BDNF are resolved from those of the receptor. Together, those studies have shown that isotope edited FTIR spectroscopy can be successfully applied to the determination of protein secondary structures of protein complexes containing either the same or different types of secondary structures. It was observed that $^{13}C/^{15}N$ uniform labeling also affects significantly the frequency of amide II′ band, which may permit the determination of hydrogen–deuterium exchange in individual subunit of protein–protein complexes.

1. Introduction

Fourier transformed infrared spectroscopy (FTIR) has been proven to be an effective technique to study secondary structures of proteins in various physical states [1,6,7,9,14]. Because the frequency of amide I band is sensitive to different type of protein secondary structure such as α-helix and β-strand, FTIR spectra of proteins have been utilized extensively in the study of conformational changes

in proteins under various experimental conditions. However, the application of FTIR spectroscopy to the investigation of protein–protein interactions has been limited by spectral overlap in the amide I region. For instance, the complex formed by two protein subunits containing the same type of secondary structure would give rise to amide I bands with complete spectral overlap. As a result, it would be essentially impossible to resolve the spectral signals from the two subunits containing the same kind of secondary structures, even with the application of resolution enhancement techniques such as Fourier deconvolution or second derivative calculation. Even for complexes with subunits containing different types of secondary structures, it can be challenging to detect conformational changes by using FTIR spectroscopy since significant band overlap often complicates the spectral assignments in the amide I/I′ region.

The usefulness of ^{13}C uniform labeling in the FTIR study of protein secondary structures has been demonstrated first by Haris and coworkers [11]. Their work have shown that by ^{13}C uniform labeling of amide C=O groups the amide I′ band of protein can be shifted to ∼40–45 cm^{-1} lower in frequency [11], and that it is thus possible to determine secondary structures of individual subunit in a protein/protein or protein/peptide complex. This method has been successfully applied to other protein/protein and protein/peptide systems [20,29]. Since ^{13}C=O groups shift amide I′ bands to approximately 40 cm^{-1} lower in frequency relative to those of ^{12}C=O amide groups [11,13,21,26], it is possible to resolve the amide I′ bands of two protein subunits in a protein/protein complex.

We have applied this technique first to a cytokine-receptor complex, i.e., G-CSF/receptor complex, in 1997 [18]. More recently, we have also investigated the structure and dynamics of a neurotrophin/receptor complex, i.e., BDNF/TrkB complex, by using isotope edited FTIR spectroscopy [17]. Our work have shown that isotope edited FTIR spectroscopy can be effectively applied to large protein–protein complexes containing either a high percentage of α-helix (G-CSF) or a high percentage of β-strand structure (BDNF). For G-CSF/receptor complex, G-CSF contains mostly α-helix and its receptor contains essentially β-strand structure. By ^{13}C/^{15}N uniformly isotope labeling of G-CSF, the major amide I/I′ band of G-CSF is shifted from near 1655 cm^{-1} to approximately 1610 cm^{-1}, which is outside the frequency range of the amide I/I′ band of the receptor. Even better spectral resolution has been achieved for BDNF/TrkB complex by using ^{13}C/^{15}N uniformly isotope labeled BDNF. Since both BDNF and its receptor TrkB contain mostly β-strand structure with amide I/I′ band centered near 1630 cm^{-1}, it is virtually impossible to separate the amide I band of BDNF from that of its receptor trkB. However, ^{13}C/^{15}N uniformly isotope labeling of BDNF shifts the amide I/I′ band of BDNF from 1630 to 1590 cm^{-1}, which makes it possible to determine the secondary structures of BDNF and trkB in the complex. Because of the dramatically improved spectral resolution, more definitive spectral assignments can be achieved in the FTIR spectra of isotope labeled ligand/receptor complexes than in those of the natural ligand/receptor complexes.

2. Results and discussion

2.1. Purification process and the yield of ^{13}C/^{15}N uniform isotope labeling

Both G-CSF and BDNF have been isolated and purified by using an *E. coli* expression system, as described previously [17,18].

Briefly, ^{13}C/^{15}N uniformly labeled G-CSF and BDNF proteins have been prepared as following. A gene coding for the recombinant human protein was transfected into *Escherichia coli* (*E. coli*) strain

possessing a temperature sensitive plasmid. $(^{15}NH_4)_2SO_4$ and $(^{13}C_6)$-glucose have been used as nitrogen and carbon isotope sources. The transfected *E. coli* strain was inoculated into medium containing $(^{15}NH_4)_2SO_4$, $(^{13}C_6)$-glucose, $MgSO_4{\cdot}7H_2O$, vitamins and trace metals in potassium phosphate buffer and was grown 24 hours with gentle shaking at 30°C. The entire culture was transferred to a 2-l sterile vessel (B. Braun) containing 1.5 l minimal medium that contained $(^{15}NH_4)_2SO_4$, $(^{13}C_6)$-glucose, $MgSO_4{\cdot}7H_2O$, vitamins and trace metals in phosphate buffer. This batch phase of the fermentation was performed at 30°C with neutralization by phosphoric acid and sodium hydroxide. Upon exhaustion of the glucose, concentrated feed containing $(^{13}C_6)$-glucose, $(^{15}NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$, vitamins and trace metals was fed into the vessel at the rate of 0.5 ml/l·OD·hr. When the culture had reached an optical density 15 at 600 nm, the cell culture was induced by increasing temperature to 40°C and the feed was replaced with another at a rate of 25 ml/l·hr. After fermentation, the cell culture was harvested and the inclusion bodies containing isotope labeled protein were collected by centrifugation. Purification of $^{13}C/^{15}N$ uniformly labeled proteins follows essentially the same procedure as that of unlabeled proteins [23,25]. The efficiency of isotope labeling has been confirmed by mass spectroscopic analysis of the isotope labeled proteins. In general, greater than 98% isotope labeling efficiency in the *E. coli* expressed proteins can be achieved, as determined by mass spectroscopy.

2.2. Complex formation between receptor and $^{13}C/^{15}N$ uniformly labeled ligand

Complexes of G-CSF/receptor and BDNF/trkB have been prepared and purified as described previously [17,18]. Briefly, the purified receptors were mixed with a slight excess of the corresponding labeled ligands in phosphate buffer at pH 7, and the mixture was incubated at 4°C overnight. The mixture was then passed through a Superdex 200 column (Pharmacia Inc.) and the column fraction containing the ligand/receptor complex was collected and concentrated accordingly. The formation of ligand/receptor complex has also been confirmed by SDS-PAGE (data not shown).

2.3. FTIR data collection and spectral analysis

After the purification of receptor/ligand complex, the complex was buffer exchanged into sodium phosphate buffer in D_2O, pD 7. Protein solutions were concentrated and H–D exchanged into 10 mM sodium phosphate, 50 mM NaCl, pD 7.0 buffer using a Centricon (Amicon) cartridge with a 10,000 Dalton molecular weight cutoff membrane. Diafiltrations of ligand, receptor and complexes in D_2O buffers were repeated several times to ensure complete removal of residual H_2O. The final protein concentrations were approximately 10 mg/ml before IR data collection and the protein solution was injected into a sample holder consisting of a pair of CaF_2 windows with a 25 μm spacer to form a uniformly thin film. Sample temperature was maintained at 20°C during IR data collection using a thermal jacket controlled by an electronic thermal controller (Boulder Nonlinear Inc.). For thermal studies, sample temperature was varied from 20 to 95°C at 5°C intervals. Typically, 1024 interferograms were co-added and Fourier-transformed to generate an absorbance spectrum at 4 cm^{-1} resolution by employing a Bomem Model MB-104 spectrometer with an INGAS detector. The spectrometer was continuously purged with a dry air system (Model 75-60, Balston Filter System, Whatman) to eliminate the spectral interference from atmospheric water vapor. Residual contributions from atmospheric vapor were digitally subtracted from the protein spectra. The broad buffer background of D_2O was subtracted from protein spectra to ensure a flat baseline in the spectral region 1400–1800 cm^{-1}.

FTIR spectra of protein samples in H_2O solutions were collected on an attenuated total reflection (ATR) device equipped with a horizontal Ge ($n = 4$) crystal plate which generates 12 reflections at 45°

angle (θ) to the incident beam (Spectra Tech Inc.). Approximately 100 μl of protein in phosphate buffer ($\sim$10 mg/ml) at pH 7.0 was evenly placed on the surface of Ge crystal during FTIR data collection. FTIR spectra were collected at 4 cm^{-1} resolution by averaging 1024 interferograms. FTIR spectral analyses including spectral deconvolution, subtraction and calculation of second derivative spectra have been carried out by using Grams/386 software (Galactic Industries Co.).

2.4. *Isotopic shift of amide* I′ *band by* $^{13}C/^{15}N$ *uniform labeling in* α*-helix and* β*-strand structures*

Figure 1 compares the second derivative FTIR spectrum of $^{13}C/^{15}N$ uniformly isotope labeled G-CSF with that of its naturally occurring counterpart (left panel, top and bottom spectra), as well as the FTIR spectrum of the isotope labeled BDNF with that of the natural protein (right panel, top and bottom). Clearly, the FTIR spectra of natural and labeled proteins are essentially identical with the exception of frequency shift. Figure 1 shows the usefulness of $^{13}C/^{15}N$ uniformly isotope labeling in both highly α-helical protein such as G-CSF and highly β-strand protein such as BDNF. Crystal structures of isolated G-CSF and BDNF reveal that there is approximately 60% α-helix and essentially no β-strand in G-CSF and that BDNF contains about 46% β-strand, 22% β-turn and no α-helix [12,24]. Peak frequency shift of amide I′ band is similar for both α-helix and β-strand structures, i.e., approximately 44 cm^{-1}. Isotopic shifts of amide I′ bands of β-turns are similar in the spectra of G-CSF and BDNF. For G-CSF, the peak frequency of amide I′ band is shifted from 1653 cm^{-1} to 1610 cm^{-1} upon $^{13}C/^{15}N$ uniform isotope labeling. The weak amide I′ bands at 1641 and 1677 cm^{-1} in the spectrum of natural G-CSF are shifted to 1599 and 1635 cm^{-1}, respectively, in the spectrum of the isotope labeled protein. For BDNF,

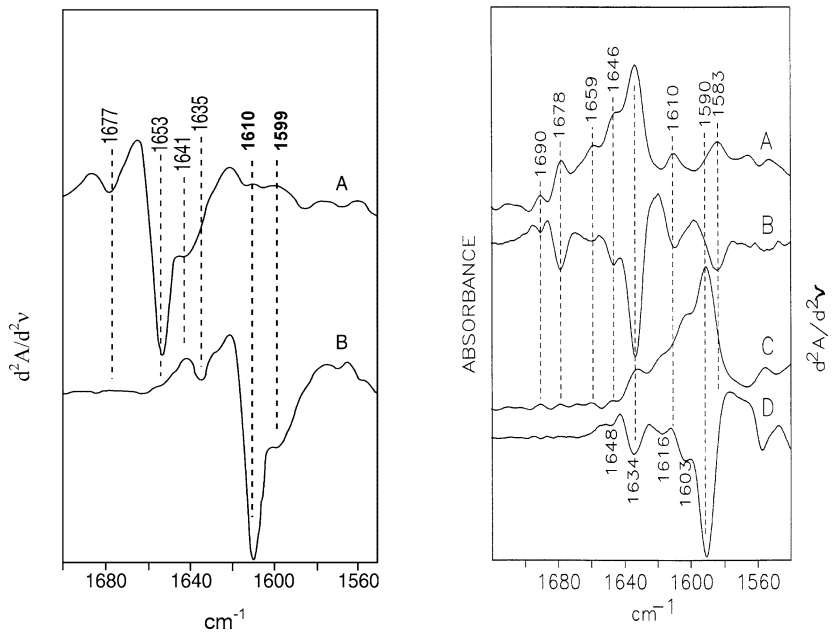

Fig. 1. Left panel: Second derivative FTIR spectra of G-CSF in D_2O buffer. Spectrum A: natural G-CSF; Spectrum B: $^{13}C/^{15}N$ uniformly labeled G-CSF. Right panel: Second derivative and Fourier deconvolved FTIR spectra of BDNF in D_2O buffer. Spectrum A: second derivative spectrum of natural BDNF; Spectrum B: Fourier deconvolved spectrum of natural BDNF; Spectrum C: second derivative spectrum of $^{13}C/^{15}N$ uniformly labeled BDNF; Spectrum D: Fourier deconvolved spectrum of $^{13}C/^{15}N$ uniformly labeled BDNF.

Table 1
Isotopic effect of ^{13}C uniform labeling on amide I′ bands

Protein secondary	Amide I′ bands		
structures	Non-labeled	^{13}C uniformly labeled	Δ (cm^{-1})
α-helix	1650–1660	1610–1620	−40
β-strand	1620–1640	1570–1590	−50
Irregular (in D_2O)	1640–1650	1590–1600	−50
Loops, Turns	1660–1690	1620–1640	−40

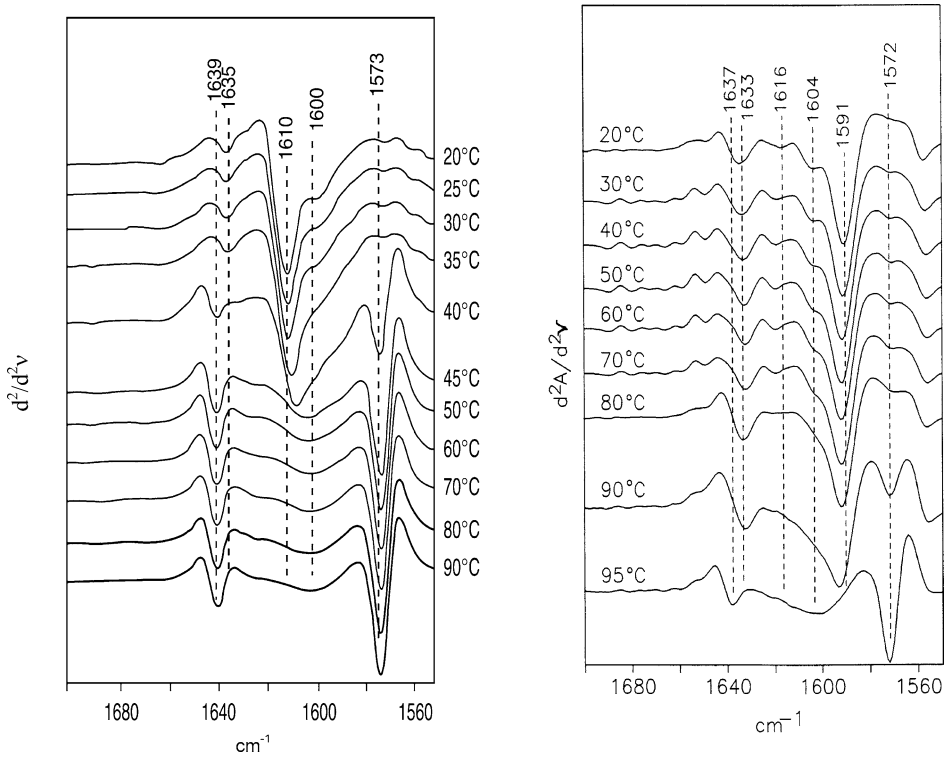

Fig. 2. Left panel: Second derivative FTIR spectra of G-CSF in D_2O buffer, at temperatures from 20 to 90°C. Right panel: Second derivative FTIR spectra of BDNF in D_2O buffer, at temperatures from 20 to 95°C.

the peak frequency of amide I′ band is shifted from 1634 to 1590 cm^{-1} in the spectrum of the isotope labeled protein. The minor amide I′ bands at 1678, 1659 and 1646 cm^{-1} are shifted to 1634, 1616 and 1603 cm^{-1}, respectively, in the spectrum of the isotope labeled protein. The frequency shift of amide I′ band observed here is excellent agreement with reported values in literature (Table 1). It is noted, however, that both G-CSF and BDNF proteins used in our study have been $^{13}C/^{15}N$ uniformly labeled while the proteins reported in literatures have been only ^{13}C uniformly labeled. This suggests that ^{15}N uniform labeling have little impact on the frequency of amide I′ band.

2.5. *Spectral interpretation and conformational analysis*

Thermal stability of $^{13}C/^{15}N$ uniformly labeled G-CSF and BDNF proteins have been investigated by following the changes in amide I′ region at temperatures from 20 to 95°C, as shown in Fig. 2. Spectral

changes in the amide I′ region of natural G-CSF and BDNF are virtually identical, with the exception of frequency shift, to those of the isotope labeled proteins at the same temperature range (data not shown). For $^{13}C/^{15}N$ uniformly labeled G-CSF, the unfolding of native α-helical conformation can be monitored by the loss of IR intensity at 1610 cm^{-1} with the increase in temperature. The loss of α-helix apparently leads to the formation of β-sheet structure at higher temperature, as evidenced by the appearance of a major IR band at 1573 cm^{-1}. At temperatures above 45°C, there seems to be a complete loss of α-helix in G-CSF in favor of β-sheet structure. Significant band broadening of amide I′ band at 1610 cm^{-1} is observed at temperature near 35°C while the IR intensity at 1573 cm^{-1} is absent at this temperature, suggesting the absence of β-sheet structure but the presence of significant conformational flexibility in G-CSF near physiological temperature.

FTIR spectra in Fig. 2 (right panel) show that the $^{13}C/^{15}N$ uniformly labeled BDNF is stable at temperatures up to 80°C, as evidenced by the invariance of the major amide I′ band at 1591 cm^{-1}. At 90°C or above, a new amide I′ band at 1572 cm^{-1} appears and is indicative of thermal denaturation of BDNF. There appears to be a complete loss in the intensity of amide I′ band at 1591 cm^{-1} at 95°C, with the appearance of a strong amide I′ band at 1572 cm^{-1} and the shift of the 1633 cm^{-1} band to 1637 cm^{-1}. This suggests that at 95°C the $^{13}C/^{15}N$ uniformly labeled BDNF suffers complete loss of native β-strand structure in favor of anti-parallel β-sheet. Interestingly, weak amide I′ bands at 1604 and 1616 cm^{-1} due to loops and β-turns lose their intensities gradually as temperature is raised from 20 to 80°C. This observation is consistent with the structural loosening or conformational flexibility of BDNF at temperature near 80°C, but still maintaining most of its native secondary structures.

Thermal denaturation of ligand–receptor complex can also be determined by using FTIR spectroscopy. However, without isotope labeling of the ligand, both ligand and receptor would give rise to an amide I′ band near 1620 cm^{-1} upon thermal denaturation. It will be thus challenging to distinguish the thermal transition of the ligand from that of the receptor within the complex. The amide I′ band overlap can be resolved completely by isotope labeling the ligand. Since the amide I′ band indicative of thermal denaturation of the isotope labeled proteins is shifted from 1620 to 1573 cm^{-1}, thermal denaturation of their corresponding receptors within the complexes can be monitored simultaneously by the amide I′ band at 1620 cm^{-1}.

Figure 3 compares the FTIR spectra of isotope labeled G-CSF/receptor (left panel) and BDNF/trkB (right panel) complexes in the temperature range from 20 to 90°C. For the $^{13}C/^{15}N$ uniformly labeled G-CSF/receptor complex at 20°C, its FTIR spectrum shows amide I′ bands at 1609, 1624, 1636, 1652, 1660, 1672, and 1686 cm^{-1}. Since the FTIR spectrum of the $^{13}C/^{15}N$ uniformly labeled G-CSF alone does not exhibit any amide I′ band with frequency higher than 1635 cm^{-1} (Fig. 1), it can be concluded that amide I bands higher than 1635 cm^{-1}, including those at 1652, 1660, 1672 and 1686 cm^{-1} in the spectrum of the complex, are due to the receptor within the complex. A number of spectral changes are evident when temperature is increased from 20 to 90°C. There is no significant IR intensity at 1573 until temperature reaches 70°C, suggesting no significant thermal denaturation of the $^{13}C/^{15}N$ uniformly labeled G-CSF in the complex at temperatures up to 70°C. This is in sharp contrast to the thermal stability of receptor-free G-CSF that exhibits significant thermal denaturation at 40°C (Fig. 2). It is therefore concluded that the native α-helical conformation of G-CSF is greatly stabilized upon binding to its receptor. This conclusion is also supported by the invariance of the amide I′ band at 1609 cm^{-1}, due to the α-helix in G-CSF, to temperature up to 70°C (Fig. 3). The amide I′ band at 1609 is shifted to lower frequency and becomes broad as temperature increases from 70 to 90°C, suggesting the thermal unfolding of α-helix in G-CSF within the complex. The appearance of a strong and sharp amide I′ band at 1573 cm^{-1} is due to the formation of β-sheet in G-CSF at temperatures above 70°C, which is

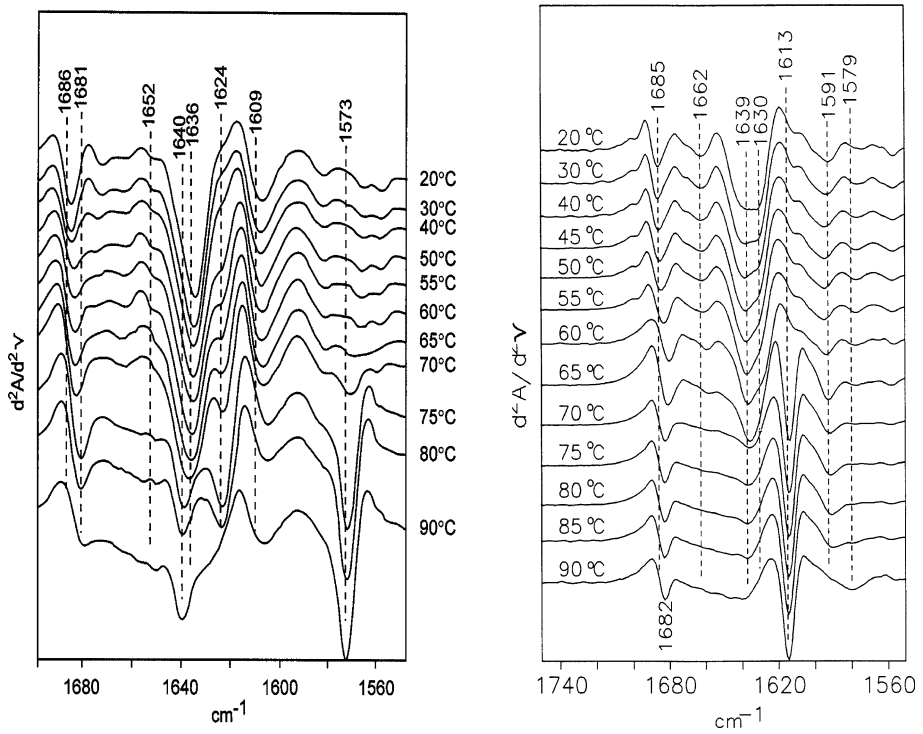

Fig. 3. Left panel: Second derivative FTIR spectra of $^{13}C/^{15}N$ uniformly labeled G-CSF/receptor complex in D_2O buffer, at temperatures from 20 to 90°C. Right panel: Second derivative FTIR spectra of $^{13}C/^{15}N$ uniformly labeled BDNF/trkB complex in D_2O buffer, at temperatures from 20 to 90°C.

consistent with the spectral changes in isolated G-CSF upon thermal denaturation (Fig. 2, left panel). In addition, the appearance of a relatively weak amide I′ band at 1640 cm^{-1} is most likely due to the thermally denatured G-CSF within the complex at temperatures above 70°C, in accordance to the FTIR spectrum of the isolated G-CSF (Fig. 2). As temperature is increased above 20°C, significant spectral changes are also observed for amide I′ bands at 1624, 1635, 1660, 1672, 1681 and 1686 cm^{-1}, which are due to the thermal denaturation of the receptor within the complex. The intensity of the amide I′ band at 1624 cm^{-1} becomes apparent at 50°C and increases significantly from 50 to 90°C, while the intensity of the major amide I′ band at 1636 cm^{-1} decreases steadily at temperatures above 50°C. The changes in amide I′ bands at 1624 and 1636 cm^{-1} are consistent with the thermal unfolding of native β-strand structure in the ligand-bound receptor at temperatures above 50°C. The frequency shift of amide I′ band at 1686 to 1681 cm^{-1} is also consistent with the structural unfolding of the receptor within the complex.

Figure 3 (right panel) also shows the FTIR spectra of the $^{13}C/^{15}N$ uniformly labeled BDNF/trkB complex at temperature range from 20 to 90°C. As reported in our previous work, FTIR data show that both BDNF and its receptor trkB contain β-strand as the predominant secondary structure [17]. The crystal structure of BDNF/neurotrophin-3 (NT-3) heterodimer revealed β-strand as the predominant secondary structure and that BDNF and NT-3 are remarkably similar in their tertiary structures [24]. The entire extracellular domain (ECD) of trkB is glycosylated and contains 398 amino acids, including a leucine-rich motif, two cysteine-rich clusters and two immunoglobulin (Ig)-like domains [8,16]. Though the three dimensional structure of the entire extracellular domain (ECD) remains to be determined, the ECD of trkB has been determined to contain mostly β-strand secondary structure [17]. The crystal structures of

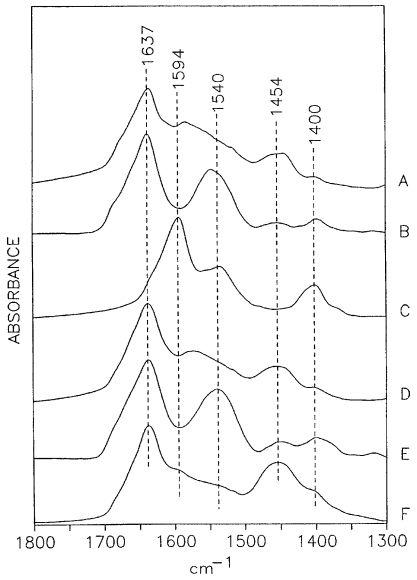

Fig. 4. FTIR spectra of BDNF in H_2O and D_2O buffers containing 10 mM sodium phosphate, 100 mM NaCl, pH or pD 7.0. Spectrum A: unlabeled BDNF in H_2O; Spectrum B: unlabeled BDNF in D_2O; Spectrum C: $^{13}C/^{15}N$ labeled BDNF in D_2O; Spectrum D: ligand-free trkB in H_2O; Spectrum E: ligand-free trkB in D_2O; Spectrum F: $^{13}C/^{15}N$ labeled BDNF/trkB complex in D_2O. FTIR spectra of proteins in H_2O solutions were collected using an out-compartment horizontal ATR device (see Experimental methods for more details).

an isolated trkB-d5 domain (the second Ig-like domain) and the complex formed between trkB-d5 and its ligand have been determined [4,28]. The determined crystal structures confirm that both ligand and the receptor contain β-strand as the predominant secondary structure [4,28]. Figure 1 (right panel) and Fig. 3 (right panel) show that amide I′ band of the $^{13}C/^{15}N$ uniformly labeled BDNF is centered at 1591 cm^{-1}, which is completely resolved from the amide I′ band (peak frequency at 1635 cm^{-1}) of its receptor trkB. The $^{13}C/^{15}N$ uniformly labeled BDNF gives rise to only a weak amide I′ band at 1634 cm^{-1} (Fig. 1), which interferes minimally with the amide I′ band of trkB. The separation of the amide I′ bands of BDNF and trkB makes it possible to monitor simultaneously the thermal stability of both components within the complex. Figure 3 (right panel) shows that native β-structure of trkB within the complex remains stable at temperatures up to 60°C, as evidenced by the major amide I′ band at 1639 cm^{-1}. At temperatures above 60°C, the intensity of amide I′ band at 1613 cm^{-1} increases dramatically and that of the 1639 cm^{-1} band decreases, indicating the thermal denaturation of trkB in the complex. However, the amide I′ band at 1591 due to the $^{13}C/^{15}N$ uniformly labeled BDNF remains unchanged at temperatures up to 85°C, suggesting that native structure of BDNF is thermal stable even though trkB is thermally denatured in the complex at temperatures above 60°C.

Figure 4 shows the FTIR spectra of BDNF, trkB and their complex in both H_2O and D_2O solutions. Amide II and II′ bands of proteins have been widely used to determine the extent of hydrogen–deuterium exchange in proteins [2,5,15,19,22,27]. H–D exchange of amide N–H group can lead to large frequency shift of amide II band from 1550 cm^{-1} to 1450 cm^{-1} [2,15,22,27]. The degree of hydrogen–deuterium exchange in BDNF, trkB and their complex has been determined by measuring the intensities of amide II and II′ bands (Table 2). Table 2 shows the intensity ratios of amide II/I and amide II′/I′ of BDNF, trkB and their complex.

Table 2
Relative intensity ratios of amide II and II′ band to those of amide I and I′ bands for BDNF, TrkB and their complex

Protein	Amide II/I (I_{1538}/I_{1638})	Amide II′/I′ (I_{1454}/I_{1638})
BDNF in H_2O	0.68	0.12
BDNF in D_2O	0.46	0.34
TrkB in H_2O	0.69	0.16
TrkB in D_2O	0.39	0.36
$^{13}C/^{15}N$ BDNF/trkB complex in D_2O	0.37	0.63

Figure 4 indicates that hydrogen–deuterium exchange is rather limited in both BDNF and trkB proteins in their isolated forms, as evidenced by the relative strong intensity of amide II band at 1540 cm^{-1} and the weak intensity of amide II′ band at 1454 cm^{-1} in the FTIR spectra of isolated BDNF (panel B) and trkB (panel E) in D_2O. Table 2 shows the measured intensity ratios, amide II/I and amide II′/I′, in consistency with the FTIR spectra in Fig. 4. The limited exchange in BDNF and trkB is consistent with the general observation that hydrogen–deuterium exchange can be extremely slow in proteins containing mostly β-sheet structure. It appears that $^{13}C/^{15}N$ uniform labeling of BDNF (Fig. 4, panel C) leads to a large frequency shift in amide II/II′ bands. FTIR spectrum of the $^{13}C/^{15}N$ uniformly labeled BDNF in D_2O shows that amide II′ band is shifted from 1454 to 1400 cm^{-1}, which produces a clear spectral window to monitor the amide II′ band of trkB in the complex. Amide II band is largely due to contribution from amide N–H in-plane deformation vibration, and is coupled with a small contribution from C–N stretching vibration [3]. This is illustrated by the large frequency shift (>100 cm^{-1}) upon deuteration of amide N–H group. Figure 4 shows that $^{13}C/^{15}N$ uniform labeling can also have significant impact on the frequency of amide II′ band, presumably due to ^{13}C and ^{15}N substitutions at amide N–H and C–N groups. Therefore, $^{13}C/^{15}N$ uniform labeling of BDNF allows the hydrogen–deuterium exchange to be determined for trkB in the complex. Figure 4 compares the FTIR spectrum of isolated trkB (panel E) with that of trkB (panel F) bound to the $^{13}C/^{15}N$ uniformly labeled BDNF. While the spectrum (panel E) of the isolated trkB in D_2O exhibits strong amide II and weak amide II′ bands, the spectrum (panel F) of trkB in the complex shows weak amide II and strong amide II′ bands. The striking difference in the intensity of amide II/II′ bands indicates that a majority of the amide group in trkB protein becomes solvent accessible when it is bound to its ligand, BDNF. It is therefore concluded that conformational flexibility of trkB increases significantly upon binding to its ligand, BDNF.

3. Conclusions

In this review, we have discussed the usefulness of uniform isotope labeling in the application of FTIR spectroscopy to investigate protein–protein interactions. Not only can $^{13}C/^{15}N$ uniform labeling of protein produce large red-shift in the frequency of amide I′ band, it also lowers significantly the frequency of amide II′ band. The frequency separation of amide II′ bands of two protein subunits makes it possible to probe the hydrogen–deuterium exchange of each subunit in protein–protein complex in which one of the subunit is $^{13}C/^{15}N$ uniformly labeled. Therefore, both protein secondary structures and conformational dynamics of protein–protein complex can be studied effectively by isotope edited FTIR spectroscopy.

References

[1] J.L.R. Arrondo, A. Muga, J. Castresana and F.M. Goni, *Prog. Biophys. Mol. Biol.* **59** (1993), 23–56.
[2] J. Backmann, C. Shultz, H. Fabian, U. Hahn, W. Saenger and D. Naumann, *Protein: Structure, Function & Genetics* **24** (1996), 379–387.
[3] J. Bandekar, *Biochim. Biophys. Acta* **1120** (1991), 123.
[4] M.J. Banfield, R.L. Naylor, A.G.S. Robertson, S.J. Allen, D. Dawbarn and R.L. Brady, *Structure* **9** (2001), 1191–1199.
[5] E.R. Blout, C. de Loze and A. Asadourian, *J. Am. Chem. Soc.* **83** (1961), 1895–1900.
[6] D.M. Byler and H. Susi, *Biopolymers* **25** (1986), 469–487.
[7] D.M. Byler and H. Susi, *J. Industrial Microbiol.* **3** (1988), 73–78.
[8] M. Haniu, J. Talvenheimo, J. Le, K. Katta, A. Welcher and M.F. Rohde, *Arch. Biochem. Biophys.* **322** (1995), 256–264.
[9] P.I. Haris and D. Chapman, *Biopolymers* **37** (1995), 251–263.
[10] P.I. Haris, D. Chapman, R.A. Harrison, K.F. Smith and S.J. Perkins, *Biochemistry* **29** (1990), 1377–1380.
[11] P.I. Haris, G.T. Robillard, A.A. van Dijk and D. Chapman, *Biochemistry* **31** (1992), 6279–6284.
[12] C.P. Hill, T.D. Osslund and D. Eisenberg, *Proc. Natl. Acad. Sci. USA* **90** (1993), 5167–5172.
[13] W. Hubner, H.H. Mantsch and H.L. Casal, *Appl. Spectrosc.* **44** (1990), 732–734.
[14] M. Jackson and H.H. Mantsch, *Critical Rev. Biochem. Mol. Biol.* **30** (1995), 95–120.
[15] H.H.J. de Jongh, E. Goormaghtigh and J.-M. Ruyshasshaert, *Biochemistry* **34** (1995), 172–179.
[16] R. Klein, L. Parada, F. Coulier and M. Barbacid, *EMBO J.* **8** (1989), 3701–3709.
[17] T. Li, J. Talvenheimo, L. Zeni, R. Rosenfield, G. Stearns and T. Arakawa, *Biopolymers* **67**(1) (2002), 10–19.
[18] T. Li, T. Horan, T. Osslund and T. Arakawa, *Biochemistry* **29** (1997), 8849–8857.
[19] H. Lenormant and E.R. Blout, *Nature* **172** (1953), 770–771.
[20] C.F.C. Ludlam, S. Sonar, C.-P. Lee, M. Coleman, J. Herzfeld, U.L. RajBhandary and K.J. Rothschild, *Biochemistry* **34** (1995), 2–6.
[21] G.V. Martinez, W.R. Fiori and G. Millhauser, *Biophys. J.* **66** (1994), A65.
[22] J.M. Olinger, D.M. Hill, R.J. Jakoben and R.S. Brody, *Biochim. Biophys. Acta* **869** (1986), 89–98.
[23] J.S. Philo, J. Talvenheimo, J. Wen, R. Rosenfeld, A. Welcher and T. Arakawa, *J. Biol. Chem.* **269** (1994), 27840–27846.
[24] R.C. Robinson, C. Radziejewski, D.I. Stuart and E.Y. Jones, *Biochemistry* **34** (1995), 4139–4146.
[25] L.M. Souza, T.C. Boone, J. Gabriloe, P.H. Lai, K.M. Zaebo, D.C. Murdock, V.R. Chazin, J. Bruszewski, H. Lu, K.K. Chen, J. Barendt, E. Platzer, M.A.S. Moore, R. Mertelsmann and K. Welte, *Science* **232** (1986), 61–63.
[26] L. Tadesse, R. Nazarbaghi and L. Walters, *J. Am. Chem. Soc.* **113** (1991), 7036–7037.
[27] J. Wantyghem, M.-H. Baron, M. Picoquart and F. Lavialle, *Biochemistry* **29** (1990), 6600–6609.
[28] M.H. Ultsch, C. Wiesmann, L.C. Simmons, J. Henrich, M. Yang, D. Reilly, S.H. Bass and A.M. de Vos, *J. Mol. Biol.* **290** (1999), 149–159.
[29] M. Zhang, H. Fabian, H.H. Mantsch and H.J. Vogel, *Biochemistry* **33** (1994), 10883–10888.