Potential of ^{13}C and ^{15}N Labeling for Studying Protein–Protein Interactions Using Fourier Transform Infrared Spectroscopy

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ABSTRACT: In this study, we examine the interaction between two bacterial proteins, namely HPr and IIA^{mtl} of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system, using FTIR spectroscopy. In an interaction involving a 1:1 molar ratio of these two proteins, when they are unlabeled, the overlap of absorbance of the amide I band arising from the peptide group vibrations of the two proteins is such that it is not possible to determine the contribution which each protein makes to the absorbance. Uniform ^{15}N labeling has little effect on the frequency of the amide I band although there is a significant shift of the amide II band. However, we show that uniform (90%) ^{13}C labeling produces a large shift of bands associated with the carbonyl moiety, especially the amide I band. This opens up windows in different regions of the infrared spectrum. Thus, when the same mixture of the two bacterial proteins is made where one of the proteins is uniformly ^{13}C-labeled (in our case HPr), the amide I maxima of this protein shifts by $\sim$45 cm^{-1} toward lower frequency and reveals the previously overlapped amide I band of the unlabeled IIA^{mtl}. This application of ^{13}C labeling shows the potential of studying protein–protein interactions using FTIR spectroscopy. With thoughtful selection of systems and labeling strategies, numerous studies with proteins should be possible. These could include, among others, enzyme–substrate and protein–ligand interactions.

Fourier transform infrared spectroscopy has proven to be a useful technique in the study of protein structure. The popularity of the technique can be clearly seen from the increasing number of publications that has appeared in recent years using this approach [see, for example, Surewicz and Mantsch (1988), Braiman and Rothschild (1988), Jackson et al. (1989), and Haris et al. (1990)]. The technique can provide information about protein secondary structure and also about subtle changes in their conformation induced by various factors such as binding of ligands. A stage has now been reached where there is a need to advance the technique to gain more specific information on protein structure and extend its application to new areas.

We have been using FTIR[1] spectroscopy to study the proteins of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). These proteins are responsible for the concomitant transport and phosphorylation of many hexoses and hexitols in bacteria [see Postma and Lengeler (1985) and Robillard and Lolkema (1988) for reviews]. The transport cycle involves the movement of the phosphoryl group from phosphoenolpyruvate through a number of transiently phosphorylated protein intermediates and finally to the transported sugar as shown in Scheme I.

Scheme I

PEP → P–EI → P–HPr → P–IIA → P–IIB → hexose–P

These proteins occur either as individual proteins or as covalent combinations of two or more proteins, depending on the system being considered. In order to determine whether the discrimination by one enzyme between phosphorylated and nonphosphorylated forms of a second enzyme is based on structural alterations, we have been examining the secondary structure of each component and looking for gross conformational changes in their structures during complex formation and phosphorylation. (This will be published in a separate communication.) During these studies, we examined proteins labeled uniformly with ^{13}C and ^{15}N. A study of these proteins revealed the potential of applying FTIR spectroscopy using uniformly ^{13}C-labeled proteins for the study of protein–protein interactions.

Materials and Methods

HPr isotopic labeling was carried out using 90% ^{13}C-enriched uniformly labeled glucose and 99% ^{15}N-enriched ammonium sulfate following the procedure of van Nuland et al. (1992). IIA^{mtl} was purified by the procedure of van Weeghel et al. (1991). Samples used in the FTIR measurements were dialyzed versus 5 mM potassium phosphate, pH 7.5, freeze-dried in 1–2-mg portions, and dissolved, before measurement, to a protein concentration of 1.77 mM and a final buffer concentration of 50 mM potassium phosphate.

FTIR spectra were recorded with a Perkin-Elmer 1750 FTIR spectrometer equipped with a TGS detector. A Perkin-Elmer Model 7300 data station was used for data acquisition and analysis. Samples were placed in a thermostated Beckman FH-01 CFT microcell fitted with a 50-μm Teflon spacer. The protein samples and their buffers were measured with identical scanning parameters after equilibration at 20 °C for 15 min. Subtraction of ^2H_2O buffer background absorption was carried out in a manner similar to that reported previously (Mitchell et al., 1988; Haris et al., 1990). Detailed analyses of the amide bands were achieved using the second-derivative procedure (Haris et al., 1986).

Results

Figure 1a shows the absorbance spectrum of the unlabeled protein HPr in ^2H_2O. The amide I maximum frequency is

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[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; Mtl, mannitol.

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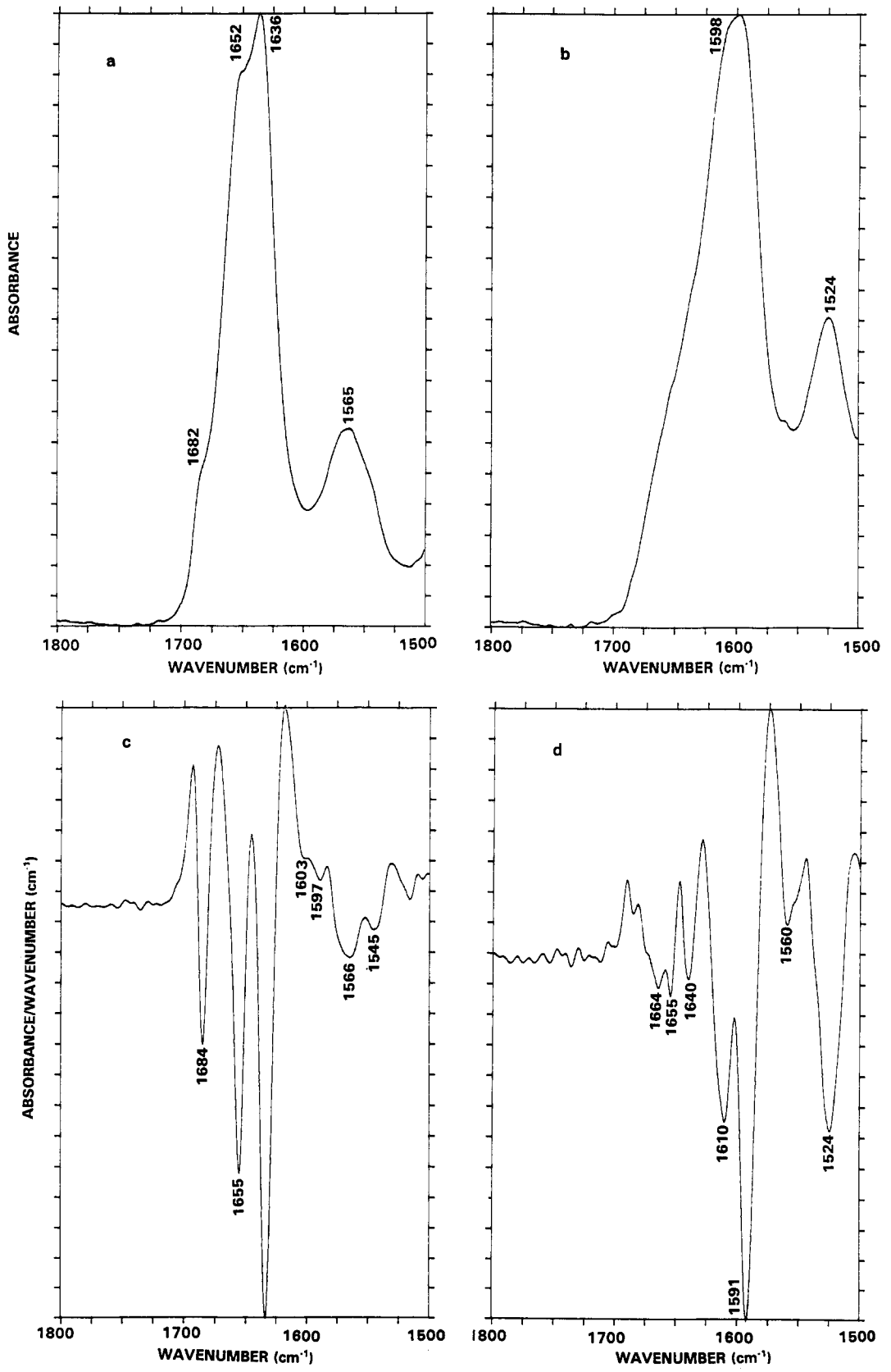

FIGURE 1: Comparison of the FTIR spectra of $^{13}C/^{15}N$-labeled and unlabeled HPr protein recorded in $^{2}H_2O$ at 20 °C. (a) Absorbance spectrum of the unlabeled HPr protein. (b) Absorbance spectrum of the labeled HPr protein. The second-derivative spectra of the unlabeled and labeled proteins are shown in panels c and d, respectively.

centered near 1636 cm^{-1}, with clear shoulders visible near 1652 and 1682 cm^{-1}. In the amide II region (near 1530–1550 cm^{-1}) there is very little absorbance. This is indicative of extensive hydrogen–deuterium exchange of the amide protons (Haris et al., 1986). The band at 1565 cm^{-1} can be attributed to the carboxylate (COO^-) groups of the protein. Figure 1b shows the absorbance spectrum of $^{13}C/^{15}N$-labeled HPr. The dramatic effect of labeling the protein can be clearly seen from the large shift that occurs in both the amide I band and the carboxylate (COO^-) band. The amide I band maximum is

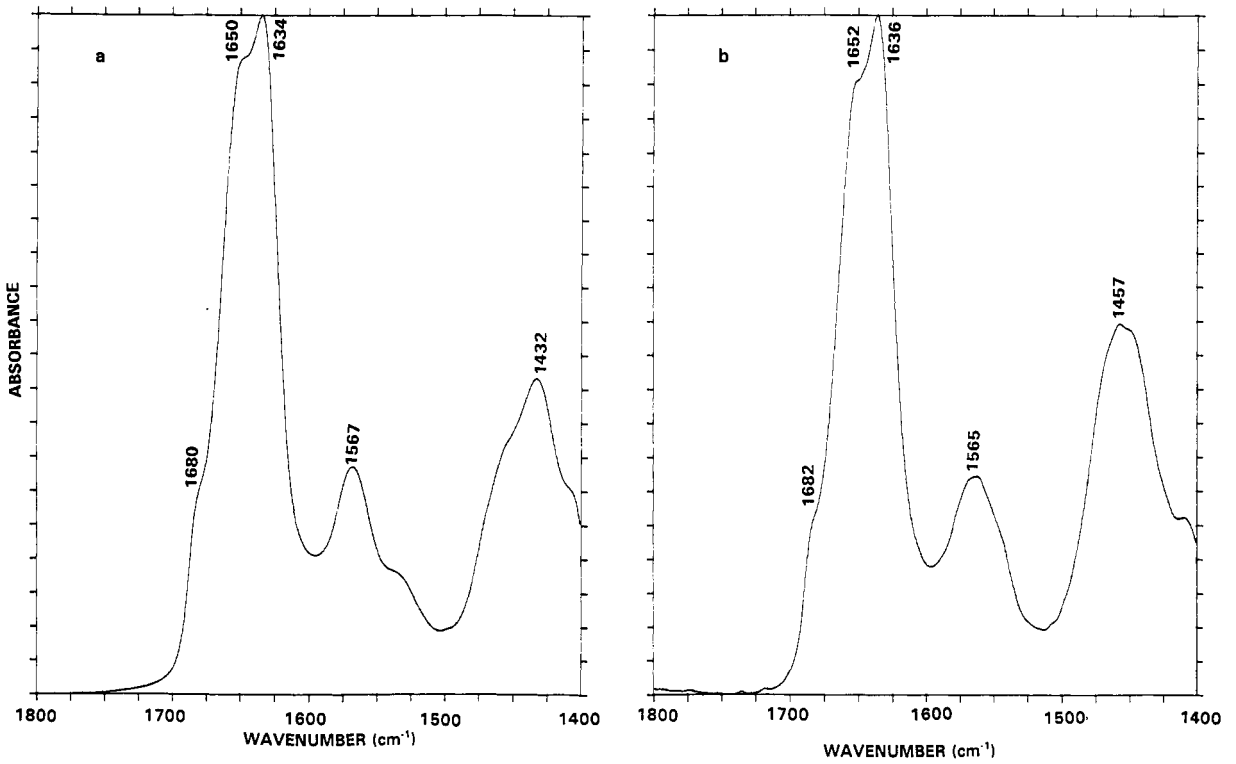

FIGURE 2: Comparison of the FTIR absorbance spectra of ^{15}N-labeled (a) and unlabeled HPr protein (b) recorded in ^2H_2O at 20 °C.

now shifted to 1598 cm^{-1}. The carboxylate band shifts from 1565 to 1524 cm^{-1}. The large shift of the amide I band leaves a window in the amide I region. The second-derivative spectra of unlabeled and labeled HPr are shown in panels c and d of Figure 1, respectively. It can be seen that the bands for the labeled proteins are similar to those of the unlabeled except that they are shifted to a lower frequency. The amide I components for the unlabeled protein are centered at 1684, 1655, and 1633 cm^{-1}. The corresponding bands for the labeled protein are centered at 1640, 1610, and 1591 cm^{-1}. Thus, all the amide I components for the labeled protein are shifted to lower frequency by approximately 42–45 cm^{-1}. It is also important to note that there is some residual absorbance seen near 1664–1655 cm^{-1}. This is probably associated with some of the unlabeled protein as only 90% ^{13}C labeling of HPr was achieved. With 100% labeling there should be no problem with an overlap of absorbance from unlabeled protein. The absorbance spectrum of HPr labeled only with ^{15}N is shown in Figure 2a. For comparison, the spectrum of the unlabeled protein is shown in Figure 2b. It can be seen that a small shift occurs in the amide I frequency as a result of ^{15}N labeling, some 2 cm^{-1} towards a lower frequency. It is noteworthy, however, that a larger shift of the amide II band occurs with ^{15}N labeling. In ^2H_2O the amide II band shifts from ~1550 to ~1450 cm^{-1}. The absorbance from H–O–^2H also occurs near 1450 cm^{-1} and hence overlaps with the shifted amide II band. However, it can be seen from Figure 2 that with the labeled protein the amide II band occurs at ~1432 cm^{-1} as compared to at 1457 cm^{-1} with the unlabeled protein.

The results presented in Figure 1 clearly show that ^{13}C labeling causes a large shift of the amide I band leaving a significant frequency range as a window for studying the interaction with other molecules which absorb in this region. Molecules which may absorb in this region include a wide range of organic and biological compounds containing carbonyl and amide groups such as drugs and ligands which interact with enzymes and receptor molecules. Furthermore, it can enable us to study protein–protein and peptide–protein interactions. The absorbance spectrum of the two unlabeled proteins (HPr and IIA^{mtl}) when they are mixed in a 1:1 M ratio is shown in Figure 3c. For comparison, the spectra of the two proteins recorded alone are presented in panels a and b of Figure 3. It can be seen that when the two unlabeled proteins are mixed (Figure 3c), the overlap is so great that it is impossible to distinguish between the absorbance of the two proteins (Figure 3a,b). However, when an equivalent mixture is made where one of the proteins, namely HPr, is labeled with ^{13}C, the amide I absorbances of the two proteins are separated and can now be clearly distinguished. This is illustrated in Figure 3d.

The second-derivative spectra of the individual proteins and the mixtures are shown in Figure 4. There is a difference in the intensity and frequency of the amide I components arising from the ^{13}C labeled protein in the mixture compared to the spectrum of the individual protein. This is a result of the fact that there is some overlap of the shifted amide I band with the weak carboxylate band (1580–1590 cm^{-1}) of the unlabeled IIA^{mtl} protein.

Discussion

FTIR spectroscopy of protein systems has been shown to be a reliable technique for the study of their secondary structure. There is however a need for greater specificity of the technique. One approach toward achieving this is to use isotopic labeling of the biomolecules. Isotopic substitution is not new to infrared spectroscopy; indeed, in the early days of infrared spectroscopic studies, protein measurements had to be conducted in ^2H_2O as H_2O absorption strongly overlapped with the protein amide I absorption. Measurements in both H_2O and ^2H_2O enable hydrogen–deuterium exchange of the peptide groups of the protein to be analyzed. This isotopic substitution of the protein also helps in identifying different types of secondary structural classes as well as in making possible the detection of subtle conformational changes in proteins (Haris et al. 1986).

FTIR spectroscopic studies have been made with lipids

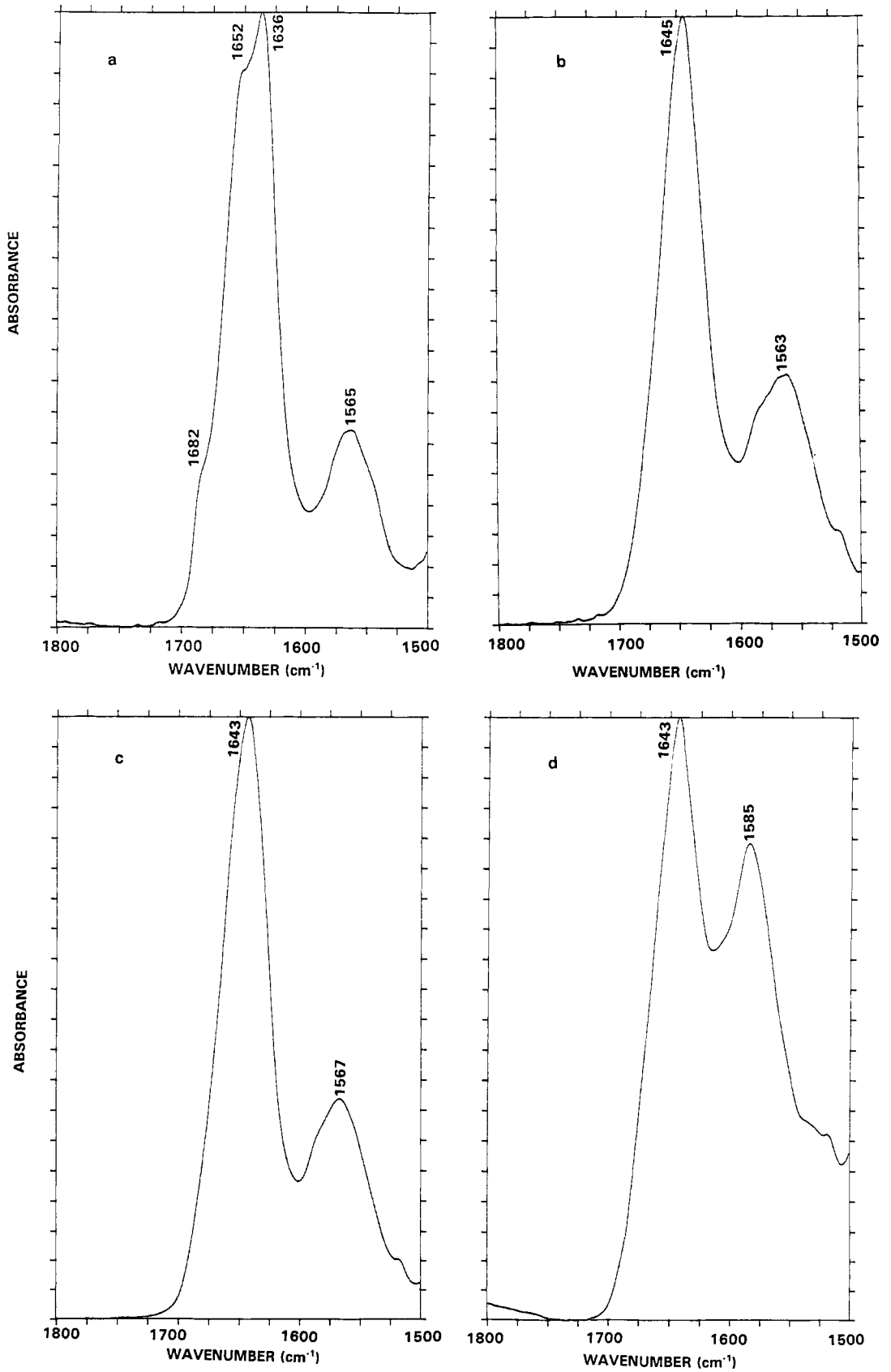

FIGURE 3: Comparison of the FTIR spectra of the HPr and the IIA^{mtl} protein when recorded alone and in an interaction involving the two proteins in a 1:1 molar ratio. The spectra were recorded for samples in ^2H_2O at 20 °C. (a) The absorbance spectrum of the unlabeled HPr protein. (b) The absorbance spectrum of the unlabeled IIA^{mtl} protein. (c) The absorbance spectrum of the HPr and the IIA^{mtl} protein mixed in a 1:1 M ratio. Both proteins are unlabeled. (d) The absorbance spectrum of the $^{13}C/^{15}N$-labeled HPr protein mixed with unlabeled IIA^{mtl} protein. (Note the separation of the two amide I maxima.)

where lipid acyl chain hydrogen atoms are substituted by deuterium atoms (Mendelsohn et al., 1984), enabling an investigation of the C–^{2}H stretching frequencies to be made without interference from amino acid side chains of proteins at high concentrations. The carbon–deuterium stretching vibration occurs at a lower band frequency. More recently,

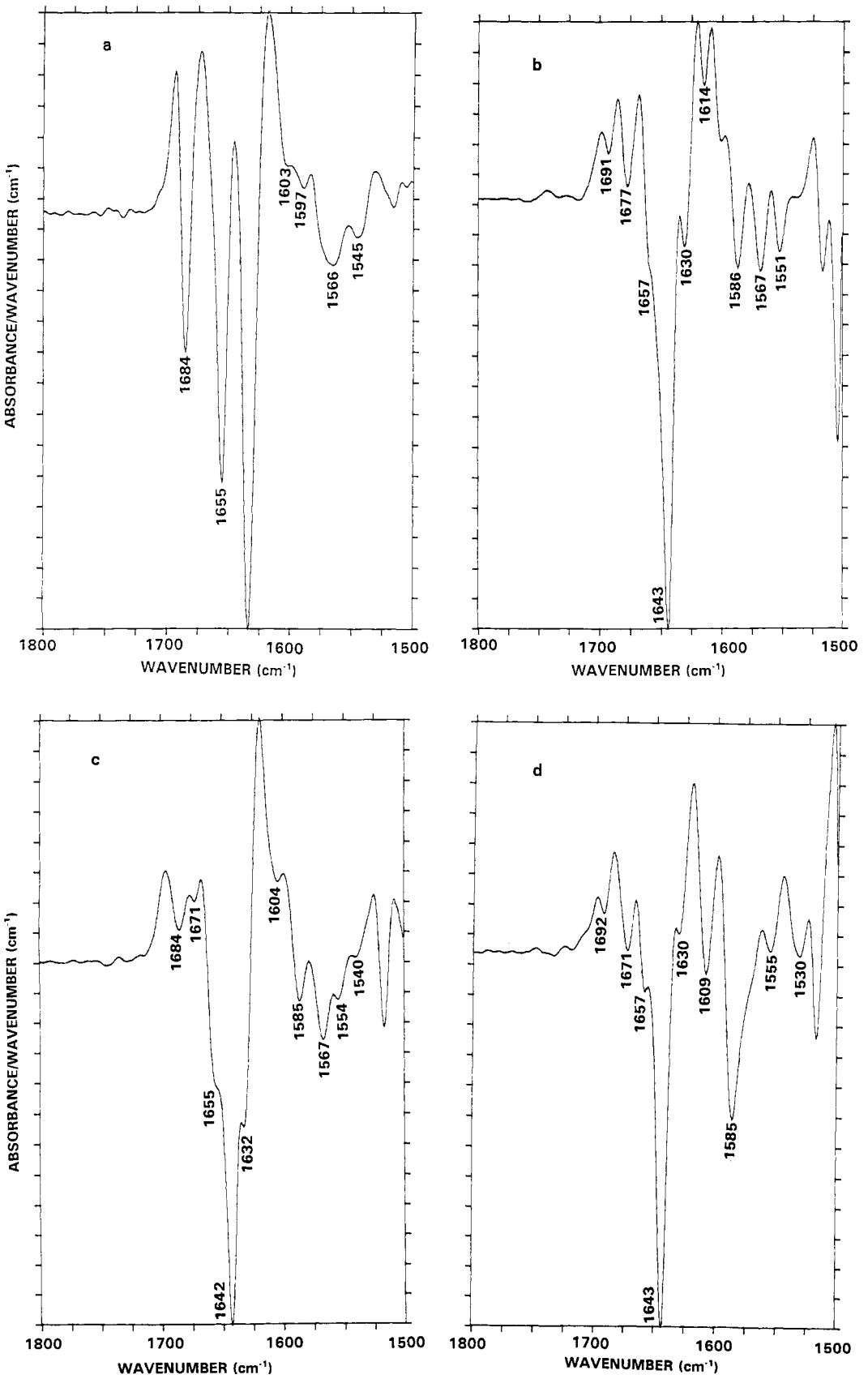

FIGURE 4: The second-derivative spectra corresponding to the absorbance spectra presented in Figure 3. (a) The spectrum of the unlabeled HPr protein. (b) The spectrum of the unlabeled IIA^{mtl} protein. (c) The spectrum of the mixture of HPr and IIA^{mtl} proteins, unlabeled. (d) The spectrum of the mixture of $^{13}C/^{15}N$-labeled HPr protein mixed with unlabeled IIA^{mtl} protein.

^{13}C labeling of lipid ester carbonyl groups has been pursued. ^{13}C labeling of one of the two ester carbonyl groups results in a $^{13}C{=}O$ vibrational band shift of $\sim$44 cm^{-1} toward lower frequencies (Hubner et al., 1990). This enables a study of the two ester carbonyl groups to be made without the complexity of the overlap.

Except for bacteriorhodopsin where specific residues have been isotopically labeled and their roles in the functional cycle

of the protein have been investigated using FTIR (Braiman & Rothschild, 1988), very few studies have been conducted on isotopically labeled proteins. There are no reports on FTIR studies of uniformly $^{13}C/^{15}N$-labeled proteins. The ability to uniformly label proteins opens new areas for the application of FTIR spectroscopy.

The results presented here show the potential of applying FTIR spectroscopy to the study of uniformly isotopically labeled proteins. Uniformly ^{13}C labeling a protein results in a large shift of the amide I band and indeed other bands associated with moieties containing carbon such as the carboxylate groups. The large shift of the amide I band can be attributed to the fact that this band arises primarily from C=O with only a minor contribution from N–H and C–N vibrations. ^{15}N labeling on the other hand leads to a significant shift of the amide II band as this band arises principally from N–H bending vibration. The amide II, although less sensitive to secondary structure compared to the amide I band, is particularly useful for studying hydrogen–deuterium exchange. In this regard, the separation of the amide II absorbance of the labeled and the unlabeled protein could be useful for obtaining structural information about protein–protein interaction, e.g., the rate of hydrogen–deuterium exchange of one protein in the presence of the other.

To conclude, our study shows that the use of ^{13}C-labeled proteins provides an opportunity to study protein–protein interactions. The strategy is to isotopically label one of the proteins such that its amide I band shifts to lower frequency; the absorbance of the unlabeled protein can then be studied without any major interference from the other protein. It will be advantageous, for example, if a β-sheet protein is chosen for ^{13}C labeling because then its interaction with an unlabeled α-helical protein is likely to give the best result as their amide I maxima are normally separated by 20–25 cm^{-1} and the ^{13}C labeling will result in a further shift of ~40–45 cm^{-1}. This will result in a total separation of the amide I maxima of the two proteins by ~60–70 cm^{-1}. This separation will be adequate for detection of structural change in both of the interacting proteins, especially the unlabeled protein. The technique may also be of great benefit in the study of interaction between protein and other molecules such as protein–ligand and protein–drug interactions using the ^{13}C-labeling strategy.

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