Abstract

Group-specific isotopic labeling of proteins using stable isotopes such as $^{15}$N, $^{13}$C, or $^2$H is a widely applied method for the accurate assignment of signals in FTIR spectroscopy. In this work, we report a systematic study on the metabolic incorporation of various stable isotope-coded amino acids into proteins of interest during organism growth. Target proteins were overexpressed in Escherichia coli cells that were grown in modified M9 medium supplemented with the respective stable isotope-coded amino acid. Through state of the art mass spectrometric analyses by means of in-gel tryptic digestion, nano-reversed-phase liquid chromatography and electrospray ionization tandem mass spectrometry we were able to quantify the degree of both the incorporation and the spreading of the isotopic label into target proteins in a fast and efficient manner. Our analyses show that specificity and/or efficiency of such metabolic labeling experiments are often limited. The efficient incorporation of stable isotopes can be facilitated by use of E. coli strains auxotrophic for the respective isotope-coded amino acid. We tested various auxotrophic strains for their potential to yield specifically labeled proteins suitable for FTIR or Raman measurements and optimized the culturing conditions. As an application example of our method we show the assignment of tyrosine bands in the time-resolved difference spectrum of the GTPase reaction of Ras by use of group-specific isotopic labeling. In conclusion, our work provides a valuable guideline for the production of group-specific isotopically labeled proteins for FTIR spectroscopic analysis.

Keywords: Proteins; Group-specific isotopic labeling; Time-resolved FTIR; Mass spectrometry; Auxotrophic strains; GTPases

1. Introduction

Application of time-resolved Fourier-transform infrared (trFTIR) difference spectroscopy can reveal detailed insight into the reaction mechanisms of proteins with atomic resolution [1]. In order to obtain accurate information about participating amino acids, H-bonding, protonation states, charge distribution and the time dependence of the reaction, bands observed in the FTIR spectra must be correctly assigned to specific groups of the protein. To this extent, group-specific isotopic labeling is an effective tool, enabling the assignment of bands obtained by FTIR spectroscopy [1–4]. In contrast to other methods such as site directed mutagenesis, this approach is non-invasive and all the changes observed in the respective FTIR spectra must have originated from the labeled groups of the protein. Nonetheless, despite the fact that isotopic labeling methods are frequently used in FTIR studies, these studies often lack an accurate, independent measure quantifying the incorporation and spreading of the label.

In order to yield group-specific isotopic labeling of expressed proteins, cells are usually grown in a modified M9 medium containing all naturally occurring amino acids except for one or more amino acids which are replaced by their labeled variants. While it is anticipated that isotope-coded amino acids are fully incorporated into expressed proteins, incomplete labeling of proteins may result from the organism’s ability to produce the selected amino acids by metabolizing unlabeled precursors. For example, in Escherichia coli, which is one of the most commonly used systems for overexpression of proteins, all amino acids are non-essential. Consequently, all
amino acids may be incompletely incorporated into proteins in the respective labeling experiments [5–7]. Furthermore, the labeled amino acid may be metabolized to other amino acids during organism growth, eventually yielding in a spreading of the label. Both incomplete incorporation of isotope-coded amino acids and spreading of the label will in all likelihood lead to the incorrect assignment of spectroscopic bands in FTIR spectra. In order to prevent such issues, an independent method for determining the degree of the incorporation and/or spreading of the label has to be sought. Since the incorporation of stable isotopes into proteins induces distinct mass shifts, mass spectrometry (MS) represents an adequate technique to monitor those labeling experiments. In previous studies radiotracers like $^{14}$C [8,9], MS after complete degeneration of the protein [10] or other spectroscopic techniques [11] were used for the estimation of the incorporation and spreading.

In this paper, we report methods for the group-specific stable isotopic labeling of target proteins using E. coli expression systems. We demonstrate the usefulness of modern MS methodologies to obtain detailed information on the degree of both incorporation and spreading of different isotopic labels into the expressed proteins. After in-gel tryptic digestion of labeled and unlabeled target proteins, the resulting peptide mixtures were individually analyzed by nano-reversed-phase liquid chromatography (nano-RPLC) directly coupled to electrospray ionization tandem mass spectrometry (ESI-MS/MS) [12]. Relative quantitative evaluation of the respective mass spectra provides detailed information on the degree of incorporation and/or spreading of the isotopic label in proteins. Additional site-specific information was obtained from the corresponding peptide fragmentation (MS/MS) spectra. We further report the adjustment of culturing conditions and the use of auxotrophic strains to yield stable isotope-labeled proteins suitable for FTIR spectroscopic investigations. Finally, we provide an applied example in which we assign bands in the difference spectra of the GTPase reaction of Ras.

2. Materials and methods

2.1. Expression and purification

Ras, Ran and Rna1p were overexpressed in different E. coli strains, namely CK600K [13] (Ras), BL21 DE3 [14] (Ran) and B834 DE3 pLysS [15] (Rna1p), with transformed ptrc c-h-ras, pET3d ran and pET11d rna1p (A. Wittinghofer, MPI Dortmund) by using an optimized M9 medium [16] consisting of thiamine–HCl (0.2 mM), thymine (0.5 mM), glucose (50 mM), MgSO$_4$ (2 mM), CaCl$_2$ (0.2 mM), Na$_2$HPO$_4$ (48 mM), KH$_2$PO$_4$ (22 mM), NaCl (12 mM), NH$_4$Cl (12.5 mM) and the appropriate antibiotics (pH 7.6). The media were used as full media, to which 0.834 mM of each amino acid was added. For metabolic labeling experiments the selected amino acid was replaced by its isotopically labeled variant. All the labeled amino acids used in this work were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). The purification of the target proteins was performed by ion exchange chromatography and gel filtration chromatography as described previously [13,17,18].

2.2. Auxotrophic strains

In addition to the above reported E. coli expression strains, auxotrophic strains were also used, namely E. coli TH16#4 [19] for glutamine, E. coli ER [20,21] for asparagine and E. coli DL39 [22] for aspartate. These strains have knocked out the genes for the synthetase(s) of the specific amino acid they are auxotrophic for, namely asnA and asnB for asparagine, glnA for glutamine, aspC and tyrB for aspartate, and gdh and gltB for glutamate [5]. All auxotrophic strains used in this work were checked for their inability to grow in the absence of the amino acid for which they should be auxotrophic for. Since ADE3 prophyase was integrated with a ADE3 lysogenization kit (Novagen®), the lysogenized host could be used to express target genes cloned in the pET vectors under the control of the T7 promoter. To ensure adequate cellular growth and protein expression, the M9 media was optimized for the strains TH16#4 and ER by using the twofold amount of isotopically labeled glutamate or asparagine, respectively. Additionally the NH$_4$Cl concentration was increased to 60 mM in the case of the ER strain. In the case of the strain DL39 the concentrations of the M9 medium were doubled and the fourfold amount glutamine was used.

2.3. Gel electrophoresis and in-gel digestion of proteins

A denatured and discontinuous SDS-PAGE [23] (10% acrylamide for Rna1p, 15% acrylamide for Ras and Ran) was performed. The gels were loaded with 1.5 μg of each labeled and unlabeled target protein. After gel electrophoresis, the proteins were visualized by Coomassie® brilliant blue G250 staining [24] and the respective bands were manually excised from the gel and then transferred into glass mini tubes. Gel pieces were immediately destained by alternately incubating them with 20 μl of 10 mM ammonium hydrogen carbonate (NH$_4$HCO$_3$) and 20 μl of 5 mM NH$_4$HCO$_3$/50% ACN, each for 10 min intervals. This step was performed thrice. Afterwards, gel pieces were dried in vacuo and either stored at −80 °C or directly subjected to proteolytic digestion with trypsin (Promega, Mannheim, Germany) dissolved in 25 mM NH$_4$HCO$_3$ (pH 7.8) at a final concentration of 0.03 μg/μl. For in-gel digestion of proteins, gel pieces were incubated overnight at 37 °C with 2 μl of trypsin solution and slight agitation. Subsequently, tryptic peptides were extracted twice with 10 μl of ACN and 5% FA mixed 1:1 (v/v); extracts were combined and ACN was removed in vacuo. For mass spectrometric analysis, samples were acidified by addition of 5% FA to a final volume of 20 μl.

2.4. Mass spectrometry

Tryptic peptides from target proteins were analyzed by nano-HPLC/ESI-MS/MS using a Dionex LC Packings system (Dionex LC Packings, Idstein, Germany) coupled to a QSTAR XL instrument (Applied Biosystems, Foster City, CA, USA). Peptide mixtures were separated by online RP capillary HPLC as previously described by Schaefer et al. [25]. The MS
instrument was equipped with a nano-electrospray ion source (SCIEX, Toronto, Canada) and distal-coated SilicaTips (FS360–20–10-D; New Objective, Woburn, MA, USA). For external calibration of the QSTAR XL instrument in the enhanced product ion mode, reserpine (m/z 609.280; Agilent Technologies, Santa Clara, CA, USA) was used. The general mass spectrometric parameters were set as follows: ion spray voltage (IS), 1800–2000 V; curtain gas (CUR), 10–14; gas 1, 1.0; declustering potential (DP), 50 V; focusing potential (FP), 220 V; declustering potential 2 (DP2), 15 V. In the Analyst QS software (Applied Biosystems, Foster City, CA, USA), the “information dependent acquisition” method was chosen, consisting of a survey MS scan (m/z 400–1200) followed by sequential isolation and fragmentation of the three most intense peaks (enhanced product ion scans, m/z 100–2000). Only multiple charged peptide ions were isolated and subjected to collision-induced dissociation using nitrogen as collision gas. Collision energies for peptide fragmentation were dynamically adjusted by the software (rolling collision energy) and previously fragmented ions were dynamically excluded for the following 16 s.

2.5. Analysis of mass spectrometric data

Efficient incorporation of stable isotopes into proteins results in a distinct mass shift, which can be directly observed in the respective MS of their proteolytic peptides. In the case that metabolic labeling of proteins by stable isotope-coded amino acids was not fully complete, the degree of isotope incorporation by

\[ n = \frac{\sum_{i=0}^{k} M_i - \sum_{i=0}^{k} A_i (C_{i-1} - B_{i-1})}{(k - j) \sum_{i=0}^{k} A_i (C_{i-1} - B_{i-1})} \]  

(2)

2.6. ATR-FTIR

For the measurements of the isolated amino acids, 200 mM solutions of the unlabeled and the labeled amino acid were prepared and measured with a horizontal diamond ATR setup (Resultec) using a Bruker IFS 88 spectrometer. Water at the respective pH was taken as the reference. A linear ATR correction was performed.

2.7. trFTIR

As an example for the application of group-specific isotopic labeling in trFTIR spectroscopy we used the intrinsic GTPase reaction of the GTPase Ras, for which we obtained a photolysis and a hydrolysis spectrum [27]:

\[ \text{Ras} \cdot \text{caged-GTP} \xrightarrow{\text{photolysis}} \text{Ras} \cdot \text{GTP} \xrightarrow{\text{hydrolysis}} \text{Ras} \cdot \text{GDP} + P_1 \]

Ras was loaded with (pHP)caged-GTP [28] according to John et al. [29]. The sample composition was 10 mM 1:1 complex of Ras with the caged nucleotide, 25 mM MgCl₂, 10 mM DTT and 200 mM Mes (pH 6). Experiments were performed at 283 K using 0.1% NF1 to catalyze the hydrolysis. This results in a much better signal-to-noise (S/N)-ratio for our measurement but does not affect the spectrum. The sample was prepared between two CaF₂ windows as detailed by Cepus et al. [30]. Photolysis of the caged compound was performed with an LPX 240 XeCl excimer laser (308 nm, Lambda Physics, Göttingen, Germany) by 12 flashes within 24 ms. More than 90% of the sample is photolyzed under these conditions. A modified Bruker IFS 66v/s spectrometer in the fast scan mode [31] was used for the measurement.

The data was analyzed between 1800 cm⁻¹ and 950 cm⁻¹ with a global fit method [32]. In this analysis, the absorbance changes \( \Delta A \) were analyzed with one exponential (\( n_t = 1 \)) with apparent rate constant \( k_1 \) and amplitude \( a_1 \):

\[ \Delta A(v, t) = \sum_{i=1}^{n_t} a_i(v) \text{e}^{-k_i t} + a_\infty(v) \]

The negative amplitude spectrum \(-a_i(v)\) is the hydrolysis spectrum; the disappearing bands face downwards and the appearing bands face upwards.

An amplitude spectrum \( a_0(v) \), calculated by

\[ a_0(v) = a_\infty(v) - \sum_{i=1}^{n_t} a_i(v) \]
3. Results and discussion

In the following section we firstly describe the workflow of our analysis. Subsequent to the experimental outline, we report the results obtained for the group-specific metabolic labeling of target proteins using stable isotope-coded amino acids, which was systematically evaluated by mass spectrometric analysis. Finally, we will illustrate the high applicability of such isotopically labeled proteins for the accurate assignment of bands in FTIR analysis.

Fig. 1 shows a schematic flow chart of our methodological approach to the group-specific labeling of proteins for FTIR analysis. Target proteins were expressed in *E. coli* using a modified M9-medium [16] containing all amino acids one of which was specifically labeled with stable isotopes. After metabolic labeling and protein purification using ion exchange chromatography and gel filtration, both the degree of the incorporation of stable isotopes into target proteins and the potential spreading of the label into other amino acids were determined by nano-RPLC/ESI-MS/MS analyses. We usually considered that stable isotope-labeled proteins were well suited for FTIR measurements if the incorporation of the label was greater 90%, and the spreading was less than 10%. In such cases the unispecifically labeled or non-labeled protein variants contribute to less than 10% each, it can be expected that they yield only minor signals in FTIR spectra. In the event that these labeling requirements were not met, media were refined and/or specific auxotrophic strains were used to obtain adequate group-specific labeling of target proteins (Fig. 1).

3.1. Successful incorporation

As an example of the complete incorporation of isotope-coded amino acids into proteins, we report the metabolic labeling of bacterially expressed Ras protein using ring-2H4-tyrosine. As a control, Ras protein was expressed under the same conditions using *E. coli* grown in medium containing tyrosine in its natural form. Following protein purification and sample preparation as described in Section 2, tryptic digests of both unlabeled and ring-2H4-tyrosine-labeled Ras were subjected to nano-RPLC/ESI-MS/MS analysis in order to assess the efficiency as well as the specificity of the labeling. Fig. 2A shows the doubly charged tryptic peptide SFEDIHQYR from Ras protein observed at a mass-to-charge (*m/z*) ratio of 597.8 (monoisotopic peak) in the respective MS spectrum. The natural isotopic peaks (13C) of the peptide are observed at higher *m/z* values, each showing a mass shift of *m/z* = 0.5. Due to isotopic labeling, a mass shift of 4 Da was introduced into the corresponding tryptic peptide from ring-2H4-tyrosine-labeled Ras protein. Accordingly, the doubly charged peptide
Information about amino acids (aa), stable isotope labels, *Tyrosine* U-13C9, *Glycine* U-13C2, *Glutamine* U-13C5, *Aspartate* U-13C4, *Threonine* U-13C4, *Proline* U-15N CK600K, *Asparagine* U-13C4, 599.8, while virtually no signal was observed at incorporation of the label (Fig. 2B). The specificity of the (unlabeled peptide form), indicating the almost complete metabolic incorporation of ring-2H4-tyrosine into Ras protein such as13C15N-threonine, which can be metabolically converted into other amino acids for the group-specific labeling of Ran protein. Since it is well known that threonine can readily be transformed to serine in bacterial metabolism, one has to consider the potentiality of spreading of the label into serine during protein synthesis and turnover. We therefore examined MS spectra of tryptic peptides from unlabeled Ran and 13C4,15N-threonine-labeled Ran, which contained either threonine or serine in their amino acid sequences. Evaluation of the respective mass spectra of tryptic peptides containing threonine but no serine indicated almost complete incorporation (>95%) of the label into Ran protein (data not shown). We next inspected the MS spectra of the doubly protonated tryptic peptide NLQYDYDISK ([M + 2H]2+ = 607.8 Da) originating from unlabeled and 13C4,15N-threonine-labeled Ran protein. In comparison with the control the isotopic pattern of the peptide from 13C4,15N-threonine-labeled Ran protein was clearly shifted to higher masses, reflecting a modified isotope distribution (Fig. 3A and B). This finding indicated the spreading of the label by conversion of 13C4,15N-threonine into serine, which was further confirmed by evaluation of the corresponding peptide MS/MS spectra (Supplementary Fig. 2). Using Eq. (2), the degree of metabolic conversion of threonine to serine was estimated to be 30% under the conditions applied in this work. In order to reduce the degree of spreading, we increased the amount of serine added to the M9 medium by 10-fold when using 13C4,15N-labeled threonine for the group-specific labeling of Ran protein. As a result, spreading of the label into serine could be sufficiently reduced (<10%) facilitating the production of 13C4,15N-threonine-labeled Ran protein suitable for FTIR analysis. Using the same approach, the spreading of the label into glycine was quantified to be 5% and no spreading into isoleucine was found.

3.2. Spreading of the label

We further investigated the capability of using amino acids, such as 15C,15N-threonine, which can be metabolically converted into other amino acids for the group-specific labeling of Ran protein. Since it is well known that threonine can readily be transformed to serine in bacterial metabolism, one has to consider the potentiality of spreading of the label into serine during protein synthesis and turnover. We therefore examined MS spectra of tryptic peptides from unlabeled Ran and 13C4,15N-threonine-labeled Ran, which contained either threonine or serine in their amino acid sequences. Evaluation of the respective mass spectra of tryptic peptides containing threonine but no serine indicated almost complete incorporation (>95%) of the label into Ran protein (data not shown). We next inspected the MS spectra of the doubly protonated tryptic peptide NLQYDYDISK ([M + 2H]2+ = 607.8 Da) originating from unlabeled and 13C4,15N-threonine-labeled Ran protein. In comparison with the control the isotopic pattern of the peptide from 13C4,15N-threonine-labeled Ran protein was clearly shifted to higher masses, reflecting a modified isotope distribution (Fig. 3A and B). This finding indicated the spreading of the label by conversion of 13C4,15N-threonine into serine, which was further confirmed by evaluation of the corresponding peptide MS/MS spectra (Supplementary Fig. 2). Using Eq. (2), the degree of metabolic conversion of threonine to serine was estimated to be 30% under the conditions applied in this work. In order to reduce the degree of spreading, we increased the amount of serine added to the M9 medium by 10-fold when using 13C4,15N-labeled threonine for the group-specific labeling of Ran protein. As a result, spreading of the label into serine could be sufficiently reduced (<10%) facilitating the production of 13C4,15N-threonine-labeled Ran protein suitable for FTIR analysis. Using the same approach, the spreading of the label into glycine was quantified to be 5% and no spreading into isoleucine was found.

Another possibility to impede spreading of the label is the use of only partly isotope-coded amino acids. For example, since arginine is a precursor for proline synthesis, we employed arginine that is labeled only at the side chain by 15N, which is then cleaved off in the corresponding metabolic pathway. Accordingly, no spreading of the label from 15N-glutamine into proline within proteins was observed as indicated in Table 1. Similarly, we used 15N-glutamine instead of uniformly labeled U-13C4,15N-glutamine to prevent spreading of the label into glutamate and/or aspartate residues within expressed proteins in *E. coli* TH16#4 (Table 1).

3.3. Auxotrophic strains

As an example of the incomplete metabolic incorporation of stable isotopes into proteins, we report the labeling of
bacterially expressed Rna1p protein using $^{13}C_4^{15}N_2$-asparagine. In such cases the degree of label incorporation should be significantly increased by using the respective auxotrophic strains of the bacteria. Fig. 4 shows the MS spectra of the tryptic peptide LENIGLQTLR ($[M + 2H]^{2+} = 578.84$ Da) of Rna1p in its native form (A) as well as originating from $^{13}C_4^{15}N_2$-asparagine-labeled Rna1p either expressed in an E. coli non-auxotrophic strain (B) or a mutant strain auxotroph for asparagine (C). Metabolic labeling using $^{13}C_4^{15}N_2$-asparagine should result in a mass shift of 6 Da, resulting in a monoisotopic mass of 581.84 amu of the doubly charged tryptic peptide LENIGLQTLR. However, expression and metabolic labeling of Rna1p protein in the E. coli non-auxotrophic strain B834 DE3 pLysS resulted in only about 20% incorporation of isotopically labeled asparagine (Fig. 4B). In order to significantly increase the level of incorporation (>95%) of the isotopically labeled asparagine as indicated by the intensities of the monoisotopic peaks of the unlabeled and labeled peptide LENIGLQTLR at $m/z$ 578.86 and 581.85, respectively (Fig. 4C). The peak observed at $m/z$ 581.56 in the spectrum results from $^{14}N$ and $^{12}C$ impurities in the supplied $^{13}C_4^{15}N_2$-asparagine ($98\%^{13}C,^{15}N$).

Note, that we tried several auxotrophic strains for each case of incomplete group-specific labeling of target proteins. As detailed in Section 2, we found that the E. coli strain ER for asparagine, DL39 for aspartate and TH16#4 for glutamine provided the best protein yields and the best degree of labeling within our modified M9 medium, respectively.

After employing MS/(MS) to ensure the correct group-specific labeling of the target protein, the isotopically labeled protein can be used for FTIR measurements. This can lead to band assignments in the spectra, which allow conclusions on the reaction mechanism as exemplified in the following section.
3.4. Band assignment in FTIR difference spectroscopy

For a pure stretching vibration of two atoms with the masses $m_1$ and $m_2$ without coupling the position of the band of the labeled protein $\nu_{\text{iso}}$ relative to the position of the band of the unlabeled protein $\nu$ can be approximated by

$$\nu_{\text{iso}} = \nu \sqrt{\frac{\mu}{\mu_{\text{iso}}}} \quad \text{with} \quad \mu = \frac{m_1 m_2}{m_1 + m_2}$$

This gives a good estimate, e.g. for an uncoupled C=O vibration at 1700 cm$^{-1}$ ($\mu \approx 6.86$) which shifts upon $^{13}$C labeling ($\mu_{\text{iso}} \approx 7.17$) by about 40 cm$^{-1}$.

For many other vibrations it is more difficult to estimate the shifts. For example the most intense bands of tyrosine are coupled vibrations with CC-stretching, CH-bending and CO-stretching character and thus the shift upon $^{13}$C- or $^2$H-labeling is lower due to participation of unlabeled atoms. Due to decoupling even shifts to higher wave numbers are possible. Thus we measured the unlabeled and the labeled form of the isolated amino acids dissolved in water. In Fig. 5A, the spectra of unlabeled, $^{13}$C$_9$-$^{15}$N-labeled and ring-$^2$H$_4$-labeled tyrosine at

Fig. 5. (A) IR-spectra of tyrosine (blue), $^{13}$C$_9$-$^{15}$N-tyrosine (red) and ring-$^2$H$_4$-tyrosine (orange) at pH 12. (B) IR-spectra of tyrosine (black) and ring-$^2$H$_4$-tyrosine (orange) at pH 1. (C) IR-difference spectra of the photolysis of unlabeled (black) and U-$^{13}$C$_9$-$^{15}$N-tyrosine labeled (red) Ras-cagedGTP. Positive bands are due to the Ras-GTP state, negative bands are due to the Ras-cagedGTP state. Due to an environmental change the two most intense bands of tyrosine appear in the difference spectrum. The band positions are similar to the isolated tyrosine with the protonated side chain as shown in (B). The shifts upon labeling are in agreement with the shifts found for the isolated amino acid. (D) The same IR-difference spectra as in (C) but with unlabeled (black) and ring-$^2$H$_4$-tyrosine labeled (orange) Ras-cagedGTP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Ras are at 1514 cm\textsuperscript{-1}. We found that by \textsuperscript{13}C\textsubscript{9}\textsuperscript{15}N labeling of tyrosine these bands shift by 36 cm\textsuperscript{-1} and 28 cm\textsuperscript{-1}, respectively. In the case of the ring-\textsuperscript{2}H\textsubscript{4} labeling of tyrosine the corresponding shifts are 80 cm\textsuperscript{-1} and 31 cm\textsuperscript{-1}. Since costs for the latter labeled amino acid are much lower and it induces even larger shifts, we recommend ring-\textsuperscript{2}H\textsubscript{4}-tyrosine for use in FTIR difference spectroscopy.

In Fig. 5B the spectra of native tyrosine and ring-\textsuperscript{2}H\textsubscript{4}-labeled tyrosine at pH 1 are shown. Due to the protonation of the side chain, the \(\nu(CCc)\) band shifted towards 1518 cm\textsuperscript{-1} and the \(\nu(CO) + \nu(CC)\) band shifted towards 1247 cm\textsuperscript{-1}. Interestingly, the band shifts due to the ring-\textsuperscript{2}H\textsubscript{4}-label are also affected by the protonation. The protonation state of tyrosine can thus be easily determined by both band position and isotopic shift using FTIR spectroscopy.

We further performed trFTIR measurements of Ras as described earlier\,[27,30,34] using both unlabeled Ras and that was labeled with either of the two different stable isotope-labeled tyrosine variants. The corresponding photolysis spectra and the comparison with unlabeled Ras are shown in Fig. 5C and D. Indeed, we found changes just in the region of the two tyrosine bands. A double band in the unlabeled spectrum at 1523 cm\textsuperscript{-1} and 1514 cm\textsuperscript{-1} is shifted towards 1488 cm\textsuperscript{-1} and 1478 cm\textsuperscript{-1} by the \textsuperscript{13}C\textsubscript{9}\textsuperscript{15}N-tyrosine labeling (Fig. 5C) and towards 1429 cm\textsuperscript{-1} and 1412 cm\textsuperscript{-1} by the ring-\textsuperscript{2}H\textsubscript{4}-tyrosine labeling (Fig. 5D). Furthermore, a negative band at 1240 cm\textsuperscript{-1} is shifted to 1212 cm\textsuperscript{-1} by the \textsuperscript{13}C\textsubscript{9}\textsuperscript{15}N-tyrosine labeling and to 1216 cm\textsuperscript{-1} by the ring-\textsuperscript{2}H\textsubscript{4}-tyrosine labeling.

3.5. Implications for the reaction mechanism

In our example we assigned the tyrosine vibration of Ras (Fig. 5A–D) and consequently information on the reaction mechanism of the GTPase reaction can now be gained. We firstly examine the negative bands, which are from the mechanism of the GTPase reaction can now be gained. We found that by \textsuperscript{13}C\textsubscript{9}\textsuperscript{15}N labeling of tyrosine these bands shift by 36 cm\textsuperscript{-1} and 28 cm\textsuperscript{-1}, respectively. In the case of the ring-\textsuperscript{2}H\textsubscript{4} labeling of tyrosine the corresponding shifts are 80 cm\textsuperscript{-1} and 31 cm\textsuperscript{-1}. Since costs for the latter labeled amino acid are much lower and it induces even larger shifts, we recommend ring-\textsuperscript{2}H\textsubscript{4}-tyrosine for use in FTIR difference spectroscopy.

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The positive bands in Fig. 5C and D are bands from the Ras-GTP state after photolysis. Here the 1514 cm\textsuperscript{-1} band shifted towards 1523 cm\textsuperscript{-1} indicating that the tyrosine is still protonated but with less hydrogen bonding in a more buried environment. In the hydrolysis difference spectrum (data not shown) we observed just the opposite band pattern, the 1523 cm\textsuperscript{-1} band disappeared and the original 1514 cm\textsuperscript{-1} was formed again. Thus this band behaves just like the threonine band which we recently assigned as a marker band for the switch I movement of Ras\,[34] and we can therefore associate the 1523 cm\textsuperscript{-1} absorption with the “on” state and the 1514 cm\textsuperscript{-1} absorption with the “off” state of Ras.

One drawback of the employed metabolic labeling approach is that it is not site-specific. Using difference spectroscopy this is often not a problem, because only the bands from groups involved in the observed reaction are seen. Since this is often only the case for one specific residue of the given type, the assignment is in many cases unambiguous. If this is not the case, a combination of site-directed mutagenesis and isotopic labeling is necessary.

The above discussed examples demonstrate the capabilities of this method which we also successfully applied to the proteins NF1\,[35], Rap and RapGAP\,[36]. Publications on the important catalytic residues of GTPase activating proteins, the arginine-finger of Ras and the asparagine-thumb of Rap are in preparation.

4. Conclusion

Group-specific labeling of proteins is a powerful method for assigning bands in FTIR spectroscopy. The latter is a prerequisite to resolve reaction mechanisms in proteins at the atomic level by vibrational spectroscopy. Since the metabolic incorporation of isotope labels into target proteins can often be low in both efficiency and specificity we advocate the use of ESI-MS of proteolytic peptides to independently assess the correct and efficient labeling of proteins using stable isotopes as a fast and accurate method which is superior to conventional methods, e.g. the determination by radiotracers. The use of adequately labeled proteins then facilitates the accurate assignment of bands in FTIR spectra.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vibspect.2007.11.003.

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