A new method of phase sensitive detection in modulation spectroscopy applied to temperature induced folding and unfolding of RNase A

D. Baurecht*, I. Porth, U.P. Fringeli

Institute of Physical Chemistry, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria

Received 31 August 2001; received in revised form 3 January 2002; accepted 3 January 2002

Abstract

Modulation spectroscopy can be an adequate tool to separate a weak system response from a huge background absorption, provided the process under consideration enables a periodic external stimulation. Phase sensitive detection (PSD) is then used to demodulate the periodic system response. We introduce a new method of PSD that can be used in modulated excitation (ME)-FTIR spectroscopy without the need of a lock-in amplifier or spectrometer build-in hardware. An advantage of this method is that only standard procedures included in all FTIR instruments, such as the measurement of time-resolved spectra, are required. The principal mathematical formalism of the used PSD is described.

As an example we show phase-resolved spectra of the amide I' region obtained from temperature modulated FTIR spectroscopic experiments of RNase A. The advantage of the ME compared to a relaxation process is shown by the power of separation of overlapping absorption bands.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Infrared spectroscopy; Modulation spectroscopy; Modulated excitation; Phase sensitive detection; RNase A

1. Introduction

External control of the function of complex molecular assemblies, such as biological systems, is often paralleled by only very small structural or orientational changes within the system. As a consequence, spectroscopic investigations of structure–function relationships generally require the separation of the weak system response from a huge background absorption of the unaffected part of the system.

Modulated excitation (ME) spectroscopy, or simply modulation spectroscopy can be an adequate tool to detect such weak absorbance changes or to separate the absorbance of single components within overlapping bands. Moreover, modulation spectroscopy can result in kinetic information of the response of the excited system, provided adequate excitation frequencies can be applied [1].

Phase sensitive detection (PSD) is a widely applied technique in electronic devices used to enhance the signal to noise ratio of periodic signals. In current ME-spectroscopic experiments, PSD is mainly performed on-line during data acquisition. This may lead to complications, since according to Lambert–Beer’s law, there is no linear relation between the phase sensitive detected intensity and the induced modulation of absorbance. Moreover, with FTIR instruments that

* Corresponding author. Fax: +43-1-4277-9525.
E-mail address: dieter.baurecht@univie.ac.at (D. Baurecht).
carry out the PSD during data acquisition, ME spectroscopy must be performed in the step-scan mode. This leads to a time consuming data acquisition, a complicated phase correction of the interferogram and to the use of additional hardware and software means. The method introduced here does the PSD offline after the measurement of time-resolved spectra (sample point spectra) without the need of additional electronics. It is referred to as vector PSD, since the whole time-resolved spectra are treated like data points in conventional PSD [2]. A more detailed mathematical analysis of PSD applied to the interferogram intensity and to time-resolved spectra is given in [1,3].

The aim of this work is mainly to demonstrate the new method of PSD and the advantage of modulation spectroscopy. Results from the temperature modulated folding and unfolding process of RNase A (Baurecht et al., in preparation) are presented as an example.

2. Theory of phase sensitive detection (PSD)

ME of a reversible process leads to a periodic response of the time dependent absorbance $A(\tilde{\nu}, t)$. The analytical procedure of the PSD at a given wavenumber $\tilde{\nu}$ consists of a multiplication of $A(\tilde{\nu}, t)$ by e.g. $\sin(k\omega t + \phi_k^{\text{PSD}})$ followed by a normalized integration over the modulation period $T = 2\pi/\omega$. This will introduce an additional parameter, namely the operator controlled phase angle $\phi_k^{\text{PSD}}$ according to Eq. (1).

$$A_k^{\phi_k^{\text{PSD}}} = \frac{2}{T} \int_0^T A(\tilde{\nu}, t) \sin(k\omega t + \phi_k^{\text{PSD}}) \, dt \quad (1)$$

Applying Eq. (1) to all wavenumbers $\tilde{\nu}$ of the spectrum leads to a vector PSD where the absorbance spectra $A(\tilde{\nu}, t)$ and $A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})$ are treated like vectors. $A(\tilde{\nu}, t)$ is the time-resolved absorbance spectrum and $A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})$ is referred to as phase-resolved modulation spectrum or phase-resolved absorbance spectrum associated with the frequency $k\omega$ ($k = 1$ corresponds to the fundamental, i.e. to the frequency of excitation $\omega$) and PSD phase setting $\phi_k^{\text{PSD}}$. For the special cases $\phi_k^{\text{PSD}} = 0^\circ$ and $\phi_k^{\text{PSD}} = 90^\circ$ (Eq. (2)), $A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})$ is equivalent to the in-phase and out-of-phase components used in 2D-correlation analysis [4,5], respectively.

$$A_k^{\phi_k^{\text{PSD}}} = A_k(\tilde{\nu}) \sin[\phi_k(\tilde{\nu})] = \frac{2}{T} \int_0^T A(\tilde{\nu}, t) \cos(k\omega t) \, dt \quad (k = 1, 2, \ldots) \quad (2)$$

$$A_k^{\phi_k^{\text{PSD}}} = A_k(\tilde{\nu}) \cos[\phi_k(\tilde{\nu})] = \frac{2}{T} \int_0^T A(\tilde{\nu}, t) \sin(k\omega t) \, dt \quad (k = 1, 2, \ldots)$$

In Eq. (2), $A_k(\tilde{\nu})$ is the absolute modulation amplitude of the absorbance and $\phi_k(\tilde{\nu})$ is the corresponding phase lag. Those are the main parameters important for the interpretation of a modulation experiment and can be determined from the measured phase-resolved spectra $A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})$ and $A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})$ using Eq. (3).

$$A_k(\tilde{\nu}) = \sqrt{A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})^2 + A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})^2} \quad (3)$$

$$\sin \phi_k = \frac{A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})}{A_k}, \quad \cos \phi_k = \frac{A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})}{A_k}$$

Thus, the purpose of the PSD in modulation spectroscopy is the evaluation of phase-resolved absorbance spectra $A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})$. In our case, this information is got from the time dependent overall absorbance $A(\tilde{\nu}, t)$ by means of the algorithm (1) using the Simpson rule for a numerical integration of $n$ discrete sample point spectra $A(\tilde{\nu}, t)$

$$A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu}) = \frac{2}{T} \int_0^T A(\tilde{\nu}, t) \sin(k\omega t + \phi_k^{\text{PSD}}) \, dt$$

$$= \frac{2}{3n} \sum_{i=0}^{n-1} A(\tilde{\nu}, t_i) \sin \left( \frac{2\pi k i}{n} + \phi_k^{\text{PSD}} \right) \quad (4)$$

If the type of the PSD-input is not the absorbance $A(\tilde{\nu}, t)$, but the spectral intensity or the intensity in the FTIR-interferogram, mathematical conversions based on the physical law of light absorbance have to be applied to the PSD output $A_k^{\phi_k^{\text{PSD}}} (\tilde{\nu})$ as discussed in [3].

3. Experimental

3.1. Sample preparation and temperature modulation

Bovine pancreatic ribonuclease A (RNase A) from Sigma was dissolved in citrate/D$_2$O buffer (pD 4.2).
Table 1
Modulation parameters used in temperature modulation of RNase A

<table>
<thead>
<tr>
<th>Period (s)</th>
<th>Frequency (mHz)</th>
<th>Temperature amplitude(^{\circ}) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>108</td>
<td>9.3</td>
<td>8.2</td>
</tr>
<tr>
<td>27</td>
<td>37.0</td>
<td>1.8</td>
</tr>
<tr>
<td>13</td>
<td>76.9</td>
<td>0.42</td>
</tr>
</tbody>
</table>

\(^{\circ}\) Amplitude of the fundamental frequency of the temperature function in the sample, determined by the analysis of a calibrated water absorbance at 3100 cm\(^{-1}\). The mean temperature was 63 °C in all experiments.

After 20 min of heating at 70 °C (to allow deuterium exchange) it was lyophilized and again dissolved in pure D\(_2\)O to obtain a concentration of \(\sim 20\) mg/ml enzyme in 20 mM citrate/D\(_2\)O buffer (pD 4.2). Measurements were done in a flow-through cell for ATR (attenuated total reflection) measurements [6,7] with a ZnSe internal reflection element (number of active internal reflections \(N = 12\)), angle of incidence \(\theta = 45^\circ\), parallel polarized light). Using this technique, the sample can be almost completely surrounded by the heat exchanger. Temperature modulations with three different periods and modulation amplitudes were performed at a mean temperature \(T_m = 63\) °C (Table 1). This is the melting temperature at which half of the molecules are unfolded under similar conditions [8]. The modulated temperature function within the sample was obtained by switching alternatively the heat exchanger of the ATR cell between two thermostats running at different temperatures as described in [5].

3.2. Data processing

IR spectra were measured at a resolution of 4 cm\(^{-1}\) on a Bruker IFS66 FTIR spectrometer equipped with a liquid nitrogen cooled MCT detector. Approximately 2000 scans per sample point interferogram were coadded during 30–240 succeeding cycles (depending on the modulation period) and the spectra were calculated using Blackman–Harris 3-Term as the apodization function. The spectrometer was purged with dry and carbon dioxide free air.

The measurement of the 16 sample point spectra was synchronized with the external temperature modulation [1]. The time gap between two spectra was 1/16 of the modulation period. Therefore, the sample points of the spectra fit exactly into a stimulation period. Time-resolved absorbance spectra were calculated using the mean intensity-spectrum (average of all 16 sample point spectra) as a reference (Fig. 1a).

![Fig. 1.](image)

(a) Time-resolved and phase-resolved FTIR-ATR absorbance spectra of the amid I’ region of temperature modulated RNase A in D\(_2\)O (c = 20 mg/ml, pD = 4.2, temperature amplitude of the fundamental stimulation frequency \(\Delta T = \pm 8.2\) °C, modulation period \(\tau = 108\) s, ZnSe internal reflection element, number of active internal reflections \(N = 12\)), angle of incidence \(\theta = 45^\circ\), parallel polarized light). (a) The 16 time-resolved spectra \((\Delta \tau = \tau/16)\) were measured within the modulation period and coadded over 30 periods (sample point spectra). Absorbance spectra were then calculated using the averaged intensity-spectrum as reference. Only eight time-resolved absorbance spectra are shown (every second, marked by 1, 3, ..., 15). The strong shift of the baseline results from overlapping with modulated water absorbance. (b) Phase-resolved spectra of the fundamental frequency with \(\phi_{\text{psd}} = 0, 10, \ldots, 90^\circ\) (from bottom to top at the indicated wavenumber). The phase lag between temperature and trigger (290°) is not considered. The phase-resolved spectra of the pure buffer solution are subtracted. Note that all absorbance spectra were calculated from untreated experimental data. The achieved signal to noise ratio is in the µAU range. The mean absorbance of RNase A during the ME (constant part) is shown as dashed line downscaled by a factor of 10.
The PSD algorithm (4) was then applied to the time-resolved absorbance spectra to obtain the phase-resolved spectra. This was performed by a self-made computer program that communicates with the spectrometer software to have access to the spectral data.

Since water absorption is strongly temperature-dependent and leads therefore to prominent T-modulation spectra, phase-resolved spectra had also to be measured from pure buffer solution. These spectra were used as a reference and were subtracted from the phase-resolved spectra of the RNase solution. A smart baseline correction was necessary because of unavoidable small differences of temperature in experiments with RNase and buffer solution. The resulting phase-resolved spectra contain only the spectral information of the modulated folding and unfolding process.

Curve fitting of all phase-resolved spectra (0–170° with 10° step width) were done with the OS/2-OPUS Software (Bruker) using the Levenberg–Marquardt algorithm and Lorentzian band shapes. All other parameters (peak position, bandwidth and intensity) were allowed to be fitted by the program. The phase lags $\phi_{1,j}$ of the components $j$ were obtained by fitting the set of data $A_{1,j}^{\text{PDS}}$ to the function given by Eq. (5) as described in [5] using $\phi_i^{\text{PDS}} = 0, 10, 20, \ldots, 170^\circ$.

$$A_{1,j}^{\phi_{i}^{\text{PDS}}} = A_{1,j} \cos(\phi_{1}^{\text{PDS}} + \phi_{i,j})$$

In Eq. (5) $A_{1,j}^{\phi_{i}^{\text{PDS}}}$ is the integral of the fitted component $j$ in the phase-resolved spectrum of the fundamental frequency ($k = 1$) and the operator controlled phase angle $\phi_i^{\text{PDS}}$. This method leads to more accurate phase lags $\phi_{i,j}$ and modulation amplitudes $A_{1,j}$ compared to the use of Eq. (4) that considers only the curve fitting results of two phase-resolved spectra.

The phase lags calculated in that way are absolute phase lags related to the experiment trigger. To get relative phase lags between the temperature function and the response of a separated component, the absolute phase lag of the temperature function itself has to be considered (see Section 3.3).

### 3.3. Temperature calibration

The temperature-dependent absorbance of water was used to determine the actual sample temperature during the modulation. Stationary measurements in the range from 30 to 80 °C were used to calibrate the temperature-dependent absorbance of water. In this temperature range we found a perfect linear relationship between water absorbance at 3100 cm$^{-1}$ and sample temperature. The absolute phase lag (related to experiment trigger) and the amplitude of the fundamental frequency of the temperature modulation were then calculated from the time-resolved spectra using Eqs. (3) and (4) and the results of temperature calibration.

### 4. Results

Only the spectral features of the amid $I'$ region that is known to be sensitive for the analysis of secondary structure of RNase A [8,10–13] are presented as an example for an application of modulation spectroscopy. The time-resolved absorbance spectra (sample point spectra) measured during the ME are shown in Fig. 1a. The strong shifts of baselines result from overlapping with temperature induced periodic effects in water. Phase-resolved absorbance spectra related to the fundamental frequency ($k = 1$) of temperature excited RNase are shown in Fig. 1b. A significant better signal to noise ratio is achieved by this technique, because noise that is not correlated with the modulation frequency is rejected by PSD.
Table 2  
Peak position and bandwidth (FWHM) of components of the amid I' region of RNase A as determined by curve fitting of phase-resolved spectra ($\phi_1^{PSD} = 0°−170°$)

<table>
<thead>
<tr>
<th>$\tau = 108,s^a$</th>
<th>$\tau = 27,s^a$</th>
<th>$\tau = 13,s^a$</th>
<th>Assigned component peak position (cm$^{-1}$)/bandwidth (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak position (cm$^{-1}$) ± S.D. (cm$^{-1}$)</td>
<td>Peak position (cm$^{-1}$) ± S.D. (cm$^{-1}$)</td>
<td>Peak position (cm$^{-1}$) ± S.D. (cm$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>1585.6 ± 0.1</td>
<td>12.1 ± 1.5</td>
<td>1585.6 ± 0.6</td>
<td>14.3 ± 2.4</td>
</tr>
<tr>
<td>1609.1 ± 0.5</td>
<td>9.8 ± 0.7</td>
<td>1608.3 ± 1.8</td>
<td>9.7 ± 0.9</td>
</tr>
<tr>
<td>1632.6 ± 0.1</td>
<td>20.3 ± 0.5</td>
<td>1632.6 ± 0.2</td>
<td>19.6 ± 1.8</td>
</tr>
<tr>
<td>1667.6 ± 1.0</td>
<td>39.1 ± 1.2</td>
<td>1667.7 ± 2.2</td>
<td>38.2 ± 1.1</td>
</tr>
<tr>
<td>1679.6 ± 0.2</td>
<td>13.1 ± 0.9</td>
<td>1679.5 ± 0.6</td>
<td>14.0 ± 2.0</td>
</tr>
</tbody>
</table>

$^a\tau$: modulation period of experiment.
The result of a curve fit (amid I' region, 1700–1570 cm⁻¹) of a phase-resolved spectrum using five components is shown in Fig. 2. The standard deviations of the fitted peak positions were lower than 0.2% of the mean value in all experiments, larger variations were observed with fitted bandwidths (Table 2). Although the main components of the amid I' band are already known from time-resolved difference spectra additional components at lower wave-numbers could be detected by phase-resolved spectra (Fig. 2).

The relative phase lags \(\varphi_{1,j}\) of the five components at different modulation frequencies are shown in Fig. 3 for the fundamental frequency \((k = 1)\). Negative values in the range 0° > \(\varphi_{k,j} > -90°\) indicate that the maximum of absorbance is delayed with respect to the temperature maximum. If \(\varphi_{k,j} \approx 0\), the component is in-phase with temperature. Negative values \(\varphi_{k,j} < -180°\) show that the maximum of absorbance is delayed with respect to the temperature minimum. If \(\varphi_{k,j} \approx 180\), the component is out-of-phase with temperature. Thus only the band at 1667 cm⁻¹ is nearly in-phase with the temperature (−4° > \(\varphi_{1,1667} > -34°\)) and therefore increase with increasing temperature, while all other components are nearly out-of-phase (−182° > \(\varphi_{1,j} > -224°\)). All absolute values of the phase lags increase with increasing frequency as expected from theory [1].

5. Discussion

Although, the aim of this work was to show the high quality of phase-resolved spectra and the capabilities in band separation an attempt is done to assign the detected components within the amid I' region to changes of the protein secondary structure and to changes in side chain absorption (Table 2).

The components with peak maxima at 1633 and 1680 cm⁻¹ are known as unambiguous markers for an antiparallel \(\beta\)-sheet. The measured phase lags of both peaks are correlated and out-of-phase with temperature as expected (Fig. 3).

The integrated absolute absorbance modulation amplitudes \(A_{1,j}\) (Eq. (5)) of the \(\beta\)-sheet component at 1633 cm⁻¹ and of the component with a peak maximum at 1668 cm⁻¹ are in the same range (e.g. for \(\tau = 108\ s\); \(\int A_{1,1633} d\tilde{v} = 0.48\) and \(\int A_{1,1668} d\tilde{v} = 0.34\ AU\ cm⁻¹\), respectively). The phase lag between these components is nearly 180° (175–187°). A direct conversion of the antiparallel \(\beta\)-sheet into the component at 1668 cm⁻¹ is therefore suggested. This component is assigned to a irregular structure [8] which could also include \(\beta\)-turns regarding the peak maximum at 1668 cm⁻¹.

The two small components at 1585 and 1609 cm⁻¹ most probably result from changes in side chain absorption during the temperature modulation. Around 1585 cm⁻¹, the guanidinium group of arginine [\(v_{sa}(CN_3H_5\text{})\)] and the carboxyl groups of aspartate or glutamate show absorbance maxima [15] (Table 2). RNase A contains 4 Arg, 5 Asp, and 5 Glu. The extinction coefficients of the considered vibrational modes are in the same order of magnitude [Arg, \(v_{sa}(CN_3H_5\text{})\): 500 M⁻¹ cm⁻¹; Asp, \(v_{as}(COO\text{})\): 820 M⁻¹ cm⁻¹; Glu, \(v_{as}(COO\text{})\): 830 M⁻¹ cm⁻¹] [14,15]. Regarding the band positions of the absorbance maximum described in the literature, Arg (1586 cm⁻¹) and Asp (1584 cm⁻¹) are more likely responsible for the detected absorbance at 1585 cm⁻¹ than Glu (1567 cm⁻¹) [14,15].

The component at 1609 cm⁻¹ may be assigned to the asymmetric stretching vibration of the guanidinium
group of arginine \([\nu_{\text{as}}(\text{CN})_2\text{H}^+])\). Additionally an influence from a ring vibration of tyrosin-OH \([\nu(\text{CC}), \delta(\text{CH})]\) must be expected in this region, too. Although there are 6 Tyr and only 4 Arg in RNase A, the extinction coefficient of Arg (460 M\(^{-1}\) cm\(^{-1}\)) is significantly higher than that of Tyr–OH (\(~160\) M\(^{-1}\) cm\(^{-1}\)) \([14,15]\). Moreover, the described band position of this Arg vibration (1608 cm\(^{-1}\)) is closer to the observed component at 1609 cm\(^{-1}\) than the described band position of this Tyr–OH vibration (1612–1618 cm\(^{-1}\)) \([15]\). On the other hand, a strong response to conformational changes of an other Tyr–OH ring vibration at 1511–1517 cm\(^{-1}\) was already reported \([8,10,13]\) and also observed in our temperature modulated experiments (data not shown). Therefore, the detected component at 1609 cm\(^{-1}\) is assigned to overlapping absorptions of Arg and Tyr. The contribution of Arg, however, must be dominant as concluded from a comparison with the intensity of the band of Tyr–OH at \(~1516\) cm\(^{-1}\). This band results in a integrated modulation amplitude of \(\int A_{1,1516} \text{d}v = 0.004\) cm\(^{-1}\) featuring a molar absorption coefficient of \(\varepsilon = 500\) M\(^{-1}\) cm\(^{-1}\) \([15]\). The integrated absorbance at 1609 cm\(^{-1}\) resulted in \(\int A_{1,1609} \text{d}v = 0.01\) cm\(^{-1}\), i.e. 2.5 times more than the integrated absorbance at \(~1516\) cm\(^{-1}\). The corresponding molar absorption coefficient of this Tyr–OH vibration, however, is reported to be \(\varepsilon = 160\) M\(^{-1}\) cm\(^{-1}\), i.e. about three times smaller than the absorption coefficient at \(~1516\) cm\(^{-1}\).

The measured difference in the phase response of the components at 1586 and 1609 cm\(^{-1}\) (Fig. 3) also indicates an influence of different side chain absorptions to the detected components. If the two components only originated from modulated absorbance of the two Arg vibrations the phase response would have to be the same. The larger phase lag of the component at 1586 cm\(^{-1}\) indicates that the structural feature corresponding to this component is a successor of the structural features corresponding to the other components.

There is another interesting feature to be mentioned, namely the absence of a modulated response of the three \(\alpha\)-helices of RNase A in our experiment. Reversible folding/unfolding of helices should lead to a modulated absorption near 1650 cm\(^{-1}\) featuring the same polarity like the antiparallel pleated sheet absorption at 1633 and 1680 cm\(^{-1}\). This experimental fact can originate in: (a) there is no conformational change of the \(\alpha\)-helix in the investigated temperature range (63 \pm 10 \(^\circ\)C), (b) the conformational change of the \(\alpha\)-helix is to slow to take place in the investigated modulation periods up to 108 s, or (c) band shape and molar absorption coefficient of both \(\alpha\)-helix and random fit exactly in the 1650 cm\(^{-1}\) region. We believe that there should be at least a small difference in the spectral features (peak maximum, bandwidth, molar absorption coefficient) between the helical structure and the unfolded state. Moreover, the responses from helix and random should have a relative phase difference of about 180° (conversion of one component into the other) which would be the best condition for high sensitive detection by PSD. We conclude therefore, that under our experimental conditions, which were \(T = 63\) °C, \(\Delta T \approx \pm 10\) °C, \(\tau \approx 100\) s, and pD = 4.2, no detectable conformational change of \(\alpha\)-helices take place.

Far UV CD data give evidence that under conditions similar to ours the major part of helices remains still folded. Labhardt \([16]\) reports for RNase A at pH 6.8, 19% helix at 55 °C and 13% at 75 °C. This would correspond to about one-third of the total helix amount to be converted peak-to-peak within our temperature modulation range. More recently, Navon et al. \([17]\) have determined a helix content of 22–23% at 20 °C and still 10% at 80 °C at a pH 5. On the other hand, those results are inconsistent with results found by Panik and Winter \([13]\). They have used Fourier self deconvolution (FSD) to separate structural components of RNase A at pH 2.5 and reported an almost negligible level of ordered structures at temperatures above 70 °C. The result of a FSD depends significantly on the choice of two parameters, a procedure, which depends to a considerable extent on the judgment of the operator. Such evaluations loose obviously a part of confidence. Our data, however, are obtained by standard analytical procedures which are completely independent from the operator, i.e. they are not altered by a transform, such as FSD.

PSD in ME spectroscopic experiments as described in this article uses only standard procedures for the measurement of time-resolved spectra available in almost all FTIR instruments. Thus upgrading a conventional FTIR spectrometer into a ME-FTIR spectrometer may be performed with low cost additional equipment for synchronization of experiment and time-resolved measurement \([18]\), as long as the time resolution achieved in the rapid scan mode is sufficient.
Time resolutions up to the limit of the spectrometer electronics may be achieved by the use of more sophisticated data acquisition techniques, like step-scan time-resolved measurements [9]. The vector PSD can be applied to a set of time-resolved sample point spectra by means of a simple spectrum calculator making use of Eq. (4). Neither analog or digital lock-in amplifiers nor spectrometer build-in hardware or software are needed. The method is applicable to a wide range of modulation frequencies [9] which are only limited at the low end by the sample stability and at the high end by instrumental electronics. Moreover, the vector PSD is the only method to perform FTIR modulation experiments at low frequencies (mHz) because of the dramatic increase of measurement time when using the step-scan technique in this frequency range.

Compared to the results of conventional difference spectroscopy of time-resolved FTIR data [8], we could resolve additional side chain vibrations in the amid I’ region. This was enabled by the phase separation performed by PSD, the significantly better signal to noise ratio, and much better background compensation achieved by ME spectroscopy. Absorptions of molecular parts which are not involved in the folding process are automatically suppressed. In contrast to band separation using second or higher derivative of spectra the quantitative information of absorbance is still available in phase-resolved spectra.

Variation of the stimulation frequency enables the determination of kinetic properties of the reaction [11], as well as the validation of the fit model applied to overlapping bands. The increasing negative phase lags of all components with increasing frequency as shown in Fig. 3 is a good confirmation of the model used for curve fitting.

References

[18] OPTISPEC Rigistrasse 5, CH-8173 Neerach, Switzerland.