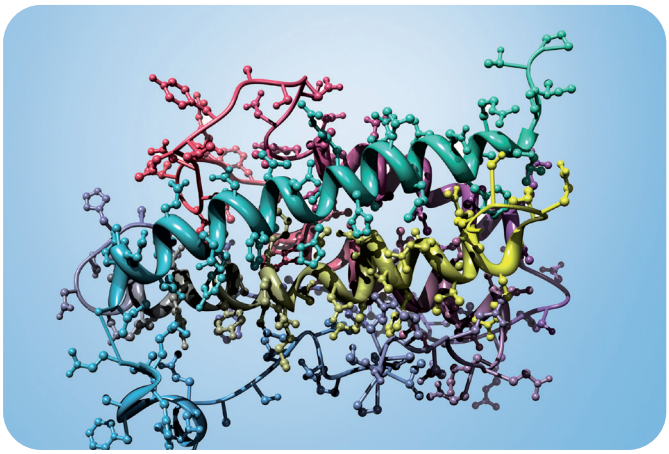

Application Note AN B404

Study of Protein Conformation with FT-IR

Similar to circular dichroism spectroscopy (CD), Fourier transform infrared spectroscopy (FT-IR) allows the determination of conformational changes of proteins (and peptides). The FT-IR technique, however, detects ß-sheet structures with a very high sensitivity. Moreover, the FT-IR spectroscopy can also be used to monitor structural changes during aggregation and fibrillation processes and even to study proteins on nano- or micro particles. In the following, the principle and the application range of this new technique in the field of protein biochemistry are described briefly.

Principle

Infrared light excites molecules to vibrate. The molecules absorb only light of certain wavelengths that corresponds to any of their specific vibration frequencies. Therefore, the position of the corresponding band in the absorption spectrum is characteristic for a certain vibration and, according to the Lambert-Beer law, the band intensity is directly proportional to the concentration of the vibrating molecule. The individual amide bonds of the proteins perform a certain vibration (C=O stretching vibration) and the energy of the amide bond is strongly influenced by the secondary structure element that contains the amide bond in question. The entire signal of all amide bonds (= number of the amino acids) within a protein produces the amide-I-absorption band. The form and the position of this band is very specific for the secondary structure of the protein. Therefore, conformational changes of the protein caused by ligand binding or changes in pH value, temperature or salt concentration are accompanied by changes of this amide-I-band in the infrared spectrum (see figure 2 and 3).

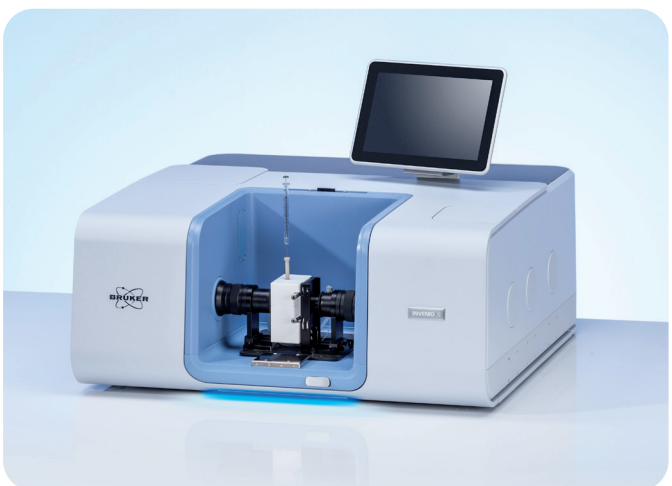

Figure 1: CONFOCHECK spectrometer system for powerful FT-IR protein analysis is based on Bruker's FTIR platform INVENIO.

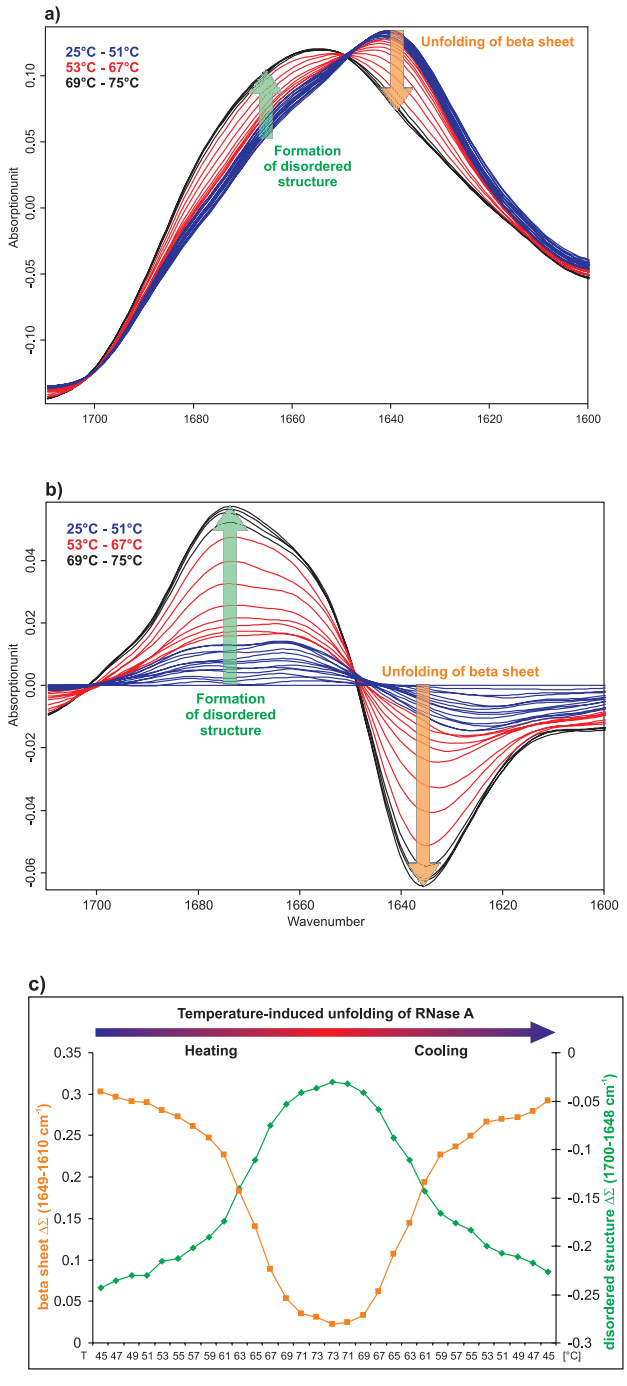

Figure 2: Unfolding of RNase A during heating. RNase A solution (10µg/µl) has been heated from 25 to 75°C in 2°C-steps. At each temperature an IR spectrum has been acquired (figure a). The change in the amide-I-band indicates the unfolding of the parallel beta-sheet content. The spectral changes become more obvious in the difference spectra in figure (b) (spectra of figure (a) minus the RNase sprectrum at 25°C). Figure (c) shows the unfolding and refolding of RNase depending on the temperature. The spectra in figure 1 and 2 have been acquired using Bruker CONFOCHECK equipped with the BioATR II cell.

Applications

The FT-IR system CONFOCHECK (Fig. 1) allows the prediction of the protein secondary structure within a minute. The protein spectrum is recorded within 30 seconds. For secondary structure prediction it is compared with the spectra of a data base using chemometric methods (e.g. Partial Least Square, Artificial Neural Networks).
The data base includes the spectra of proteins of which the secondary structure is known. However, the main application area of the FT-IR spectroscopy in the field of protein analysis is not the determination of the secondary structure, but the detection of structural changes with a high sensitivity. Using FT-IR, conformational changes can be monitored which are induced by the binding of low-molecular substances, or by changes of the pH value or the salt concentration (see fig. 3). A further typical application area is the observation of temperature- or chemically induced unfolding and refolding processes (see figure 2 and 3).

Protein formulation

Proteins become also more and more important as active drugs in pharmaceuticals. Especially therapeutic antibodies are a promising approach for the treatment of diseases that have been incurable so far. Under what conditions remains the protein stable? How long lasts this stability? These and other questions have to be answered in the course of the formulation optimization. The classical method to detect the aggregates of the protein is the size-exclusion chromatography. The denaturation process observed with this method mostly starts mechanistically at an earlier stage, namely with conformational changes of the protein and often under the formation of a typical ß-sheet structure. These denaturation processes can be monitored using the FT-IR method that allows the detection of instable formulations already at a very early stage. Also the conformational changes during the ongoing aggregation process can be observed directly with this method. To ensure a stable formulation, the proteins are often kept in complex buffers with a large number of additives like sugars, polyalcohols or amino acids and they are stored either in dissolved state (liquid formulation) or in powderform (solid formulation) after the lyophilization. The FT-IR method now gives the opportunity to measure structural changes of proteins both in liquid and in solid formulations regardless of the buffers or additives.

Aggregation and fibrillation

Neurodegenerative diseases like the Alzheimer disease or the Creutzfeldt-Jacob disease (CJD) are accompanied by protein fibrillation. Processes like aggregation and fibrillation can easily be monitored in real time using FT-IR. This method does not only allow detecting the kinetic of forming multimeric structures, but also monitoring conformational changes

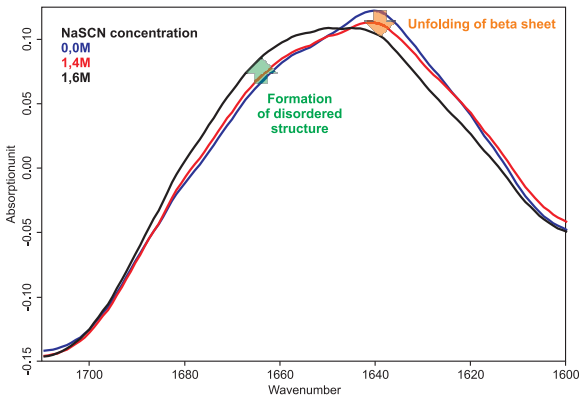

Figure 3: Unfolding of RNase A induced by salt. To unfold the protein, RNase A solution (10µg/µl), at 40°C, thiocyanate (2 M) has been dialyzed into the sample solution. During this diffusion process IR spectra have been acquired at certain thiocyanate concentration levels (0M / 1.4M / 1.6M).

in the monomers. During the protein aggregation process, very often antiparallel ß-sheet structures form which can be detected with a very high sensitivity using FT-IR.

Proteins on nano and micro particles

Due to the strong scattering of the UV-light on the particles, immobilized proteins can not be studied using the CD spectroscopy. The FT-IR spectroscopy, however, is a suitable method for analyzing proteins also bound to nano or micro particles because of the use of infrared radiation with longer wavelength. A typical question is whether the structure of the immobilized protein is intact (i.e. it corresponds to the dissolved protein) or has changed during the immobilization. Furthermore, the influence of the chemical environment (pH, buffer, salt) and the temperature on the conformation of the bound protein as well as the structurel effect of ligand binding can be studied.

Protein-ligand binding

To test the possible effect of a substance on the activity of a certain target protein, often the binding between this substance and the protein is analyzed at first. Common methods to do this are surface plasmon resonance and fluorescence correlation spectroscopy. In contrast to these techniques, FT-IR does not examine the kinetic of the protein-ligand binding but the effect of the ligand on the target protein. If the ligand binding induces a conformational change of the protein, this will probably have also an influence on the protein activity.

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