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Application Note

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Protein Complex Concentration with Vivaspin® Prior to Fourier Transform Infrared (FTIR) Spectroscopy

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Introduction

Infrared spectroscopy is a favoured method for structural and functional investigation of small molecules. A spectrum is obtained by passing infrared radiation through a given sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears, corresponds to the frequency of a vibration of a part of the sample molecule. The resulting spectrum is therefore a fingerprint of the molecule which has been measured[1].

The most significant advances in infrared spectroscopy came with the introduction of Fourier transform infrared spectrometers. These instruments employ an interferogram and exploit the well-established mathematical process of Fourier transformation, the results of which include improvement in the quality and accuracy of infrared spectra, and the time required to collect data. This means that Fourier transform infrared spectroscopy (FTIR) can be used to characterize much larger molecules, including proteins and other biological systems[2]. FTIR is a complementary technique to others, in the quest for understanding the detailed mechanisms of protein functions.

While x-ray crystallography yields the detailed structure of an essentially static state of the protein, and Reasonance Raman or optical spectroscopy provide information on the interactions of only a chromophore which is conjugated to the molecule of interest, FTIR reveals all the structural changes associated with protein reactions. In FTIR there is no prerequisite for an ordered structure, nor a limitation on molecule aggregation or size. Information can be gained on the structures of high molecular weight proteins either in solution or in a membrane-bound state. Therefore, FTIR is a technique well suited to studies of the relationship between structure and function in proteins, such as conformational changes induced by ligand or substrate binding. Furthermore, FTIR data can be examined from a kinetic perspective[1]. As a result FTIR is an important technology in biomedical and biotechnical research.

Due to the high number of atoms in a protein and its solvent, there is a large overlap of signals resulting in a spectrum consisting of only a few broad bands (Figure 1B). Such a spectrum yields valuable information at the level of secondary structure, but interpretation at the level of individual bonds is usually not feasible. A useful strategy to overcome this problem is difference spectroscopy. Using this method, a spectrum is obtained by subtraction of the spectra of a protein in two different states, and the resulting difference spectrum (Figure 1C) shows signals due to the structural changes cancelling out the bulk of the protein and solvent. Light minus dark FTIR difference spectroscopy is a powerful and highly sensitive technique for studying changes in protein conformation upon photochemical reduction. The technique was applied here to a bovine heart redox enzyme the bc_1 complex (also known as ubihydroquinone cytochrome c reductase, or Complex III). The objective was to investigate conformational changes associated with the mobile head domain of the 'Rieske' protein subunit, protonation and deprotonation reactions of amino acid residues, hydrogen bonding changes to side chains and to the backbone, and the interactions between inhibitors, substrates, prosthetic groups and the protein.

The bc_1 complex is a major component of the respiratory chain of eukaryotes and prokaryotes. Coupled electron transfer to proton translocation by a Q-cycle mechanism first described by Peter Mitchell provides the energy for the production of ATP. The protein is a dimer in its native state (500 kDa) and contains eleven subunits per monomer[3]. Photochemical reduction of the redox components of the bc_1 complex was investigated using flash induced flavin semiquinone generated from flavin mononucleotide (FMN). Only the prosthetic groups and a small number of amino acid residues out of a total of about 4,000 are involved in the confomational changes upon photochemical reduction of the bc_1 complex. The amplitude of the changes in the light minus dark difference spectrum (Figure 1C) is less than 1% of that of the absolute spectrum (Figure 1A). For this reason and due to the fact that water has a high extinction coefficient in the region of interest, FTIR requires a small volume (5-10 mL) of highly concentrated (400-470 mM) protein sample to achieve a high signal to noise ratio (sensitivity).

Materials and Methods

The beef heart cytochrome bc_1 complex was prepared according to the method described in Schägger *et al*[4] using Triton X-100 as detergent[4]. A 1 mL sample of 92 mM stock cytochrome bc_1 complex was concentrated using Vivaspin® 2 or 6 centrifugal ultrafiltration devices (50 kDa MWCO PES) to a final volume of 200 µL with concentration of 400 - 470 mM in 2 hours at 4 °C. Excess Triton X-100 and glycerol - otherwise detrimental to the attainment of a good FTIR spectrum - were removed by diafiltration. The concentration of the concentrated enzyme was determined optically (Figure 1A).

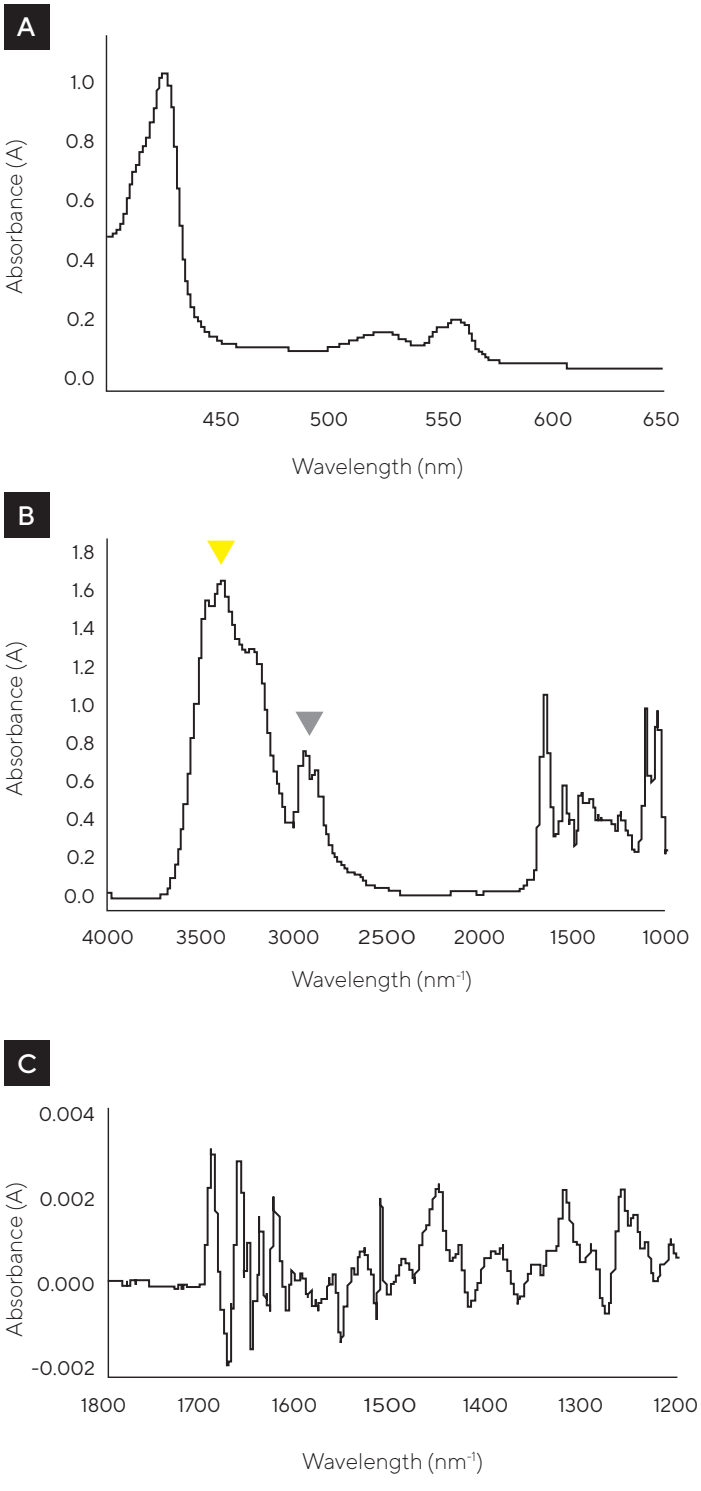
Results

Figure 1 shows the absolute visible and FTIR spectra of concentrated cytochrome bc_1 complex. The optical spectrum (panel A) was used to determine the concentration of the concentrated enzyme. The FTIR absolute spectrum (panel B) shows a bc_1 complex sample with optimal absorbance required for a high signal-to-noise FTIR difference spectrum. The amide I and II refer to protein peptide bond vibrations. Water absorbs in this region, distorting the ratio of amide I to II. By using a concentrated sample, this interference is largely removed.

The already published light minus dark FTIR spectrum (panel C) of cytochrome c was used as a control in our studies.

Figure 1.

Optical and FTIR spectra of concentrated bc_1 complex and cytochrome c. A: The absolute reduced visible spectrum 425 mM final concentration. B: The absolute oxidised FTIR spectrum. Prominent features corresponding to the protein backbone are indicated with triangles (amide 1, yellow; and amide II, grey. C: FTIR light minus dark difference spectrum of cytochrome c.



Conclusions

The speed of concentrating the target molecule in this study using Vivaspin® 2 and 6 devices in contrast to alternative products allowed a prompt evaluation and analysis of the sample by FTIR difference spectroscopy. A high signal-to-noise light minus dark FTIR difference spectrum was obtained for all the redox components investigated as a direct result of the high concentration factors achieved using Vivaspin® devices.

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