

## Concentration of a Multimeric Membrane protein complex - Protein complex sample concentration for Fourier transform infrared (FTIR) spectroscopy

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### Introduction

#### Infrared Spectroscopy

Infrared spectroscopy is a favourite method for structural and functional investigation of small molecules. It measures the vibrations of atoms. 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 spectrum obtained is a fingerprint of the entire molecular structure (1).

#### Fourier transform infrared (FTIR) spectroscopy

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 is an improvement in the quality and accuracy of infrared spectra and the time required to obtain data. Coupled with the improvements in computers, infrared spectroscopy can now be successfully used to characterise proteins and other biological systems (2).

FTIR is a complementary technique to others, in the quest for understanding the detail mechanisms of protein reactions. While X-ray crystallography yields the detailed structure of an essentially static state of the protein, FTIR reveals all the structural changes associated with protein reactions, in contrast to Resonance Raman or optical spectroscopy that yields information on the interactions of the chromophore only. In FTIR there is no prerequisite for an ordered structure or the limitation of aggregation and size.

Information can be gained on the structures of high molecular mass proteins in solutions or in the membrane bound state. FTIR is a technique well suited to studies of the relationship between structure and function in proteins i.e. issues such as ligand or substrate induced conformational changes. Furthermore, FTIR data can be examined from a kinetic perspective (1). As a result FTIR is a new emerging technology gaining popularity in the biomedical and biotechnology fields.

#### FTIR difference spectroscopy

Due to the large 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 (Fig 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. In difference spectroscopy a spectrum is obtained by subtraction of the spectra of a protein in two different states, the resulting difference spectrum (Fig 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 is applied here to a bovine heart redox enzyme the  $bc_1$  complex (also known as ubiquinone cytochrome c reductase or Complex III). The aim is 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.

#### Pre-requisite for high signal to noise FTIR difference spectrum

Only the prosthetic groups and a small number of amino acids residues out of a total of about 4000 are involved in the conformational changes upon photochemical reduction of the  $bc_1$  complex. The amplitude of the changes in the light minus dark difference spectrum is less than 1% of that of the absolute

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spectrum (Fig 1c). 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).

### The bc<sub>1</sub> complex

The bc<sub>1</sub> 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). The bovine enzyme contains eleven subunits per monomer (3). Photochemical reduction of the redox components of the bc<sub>1</sub> complex was investigated using flash induced flavin semiquinone generated from flavin mononucleotide (FMN).

### Method

The beef heart cytochrome bc<sub>1</sub> complex was prepared according to the method described in Schägger et al (4) using Triton X-100 as detergent (4). A stock cytochrome bc<sub>1</sub> complex of 1 ml of 92 mM was concentrated using a 2 or 6 ml Vivaspin 50,000 MWCO with a PES membrane to 200 µl of 400 - 470 mM in 2 hrs at 4 °C. As a result, excess Triton X-100 and glycerol otherwise detrimental to the attainment of a good FTIR spectrum, were removed. The concentration of the concentrated enzyme is determined optically (Fig 1a).

### Results

Fig 1 shows the absolute visible and FTIR spectra of concentrated cytochrome bc<sub>1</sub> complex. The optical spectrum (Fig 1a) was used to determine the concentration of the concentrated enzyme. The FTIR absolute spectrum (Fig 1b) shows a bc<sub>1</sub> complex sample with optimal absorbance required for a high signal to noise FTIR difference spectrum. The amide I and II refer to protein peptide bonds vibrations. Water absorbs in this region, distorting the ratio of amide I to II. Using a concentrated sample the distortion is largely removed. The already published light minus dark FTIR spectrum (Fig 1c) of cytochrome c was used as a control in our studies.

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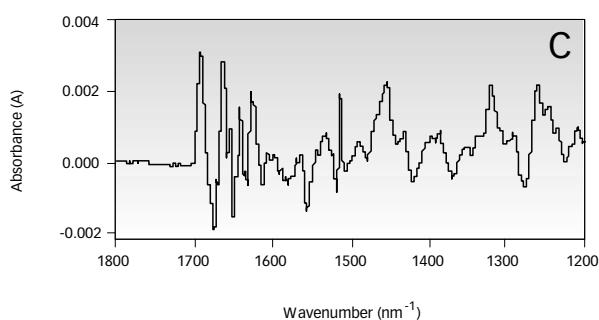
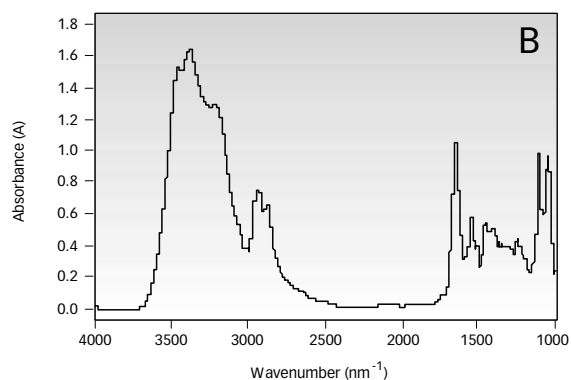
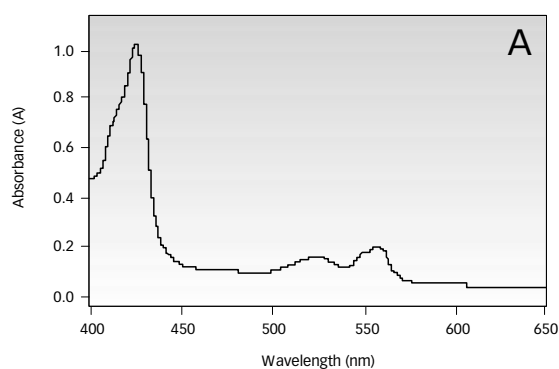
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### Fig 1: The optical and FTIR spectra of concentrated bc<sub>1</sub> complex and cytochrome c.

- The absolute reduced visible spectrum 425 mM final concentration.
- The absolute oxidised FTIR spectrum.
- FTIR light minus dark difference spectrum of cytochrome c.

### Conclusion

The speed of concentrating the protein using Vivaspin 6 devices in contrast to other devices allowed a prompt evaluation and analysis of the sample. A high signal to noise light minus dark FTIR difference spectra was obtained for all the redox components investigated as a direct result of using Vivaspin 6 devices.



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