Biochimica et Biophysica Acta 1834 (2013) 2849–2858

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

Recent applications of ATR FTIR spectroscopy and imaging to proteins

Stefanie E. Glassford [a], Bernadette Byrne [b], Sergei G. Kazarian [a,*]

[a] Department of Chemical Engineering, Imperial College London, SW7 2AZ, UK
[b] Division of Molecular Biosciences, Imperial College London, SW7 2AZ, UK

ARTICLE INFO

Article history:
Received 22 May 2013
Received in revised form 24 July 2013
Accepted 27 July 2013
Available online 6 August 2013

Keywords:
FT-IR spectroscopy
Chemical imaging
Surface-enhanced spectroscopy
Protein analysis
Total internal reflection
High throughput

ABSTRACT

Attenuated Total Reflection (ATR) Fourier Transform Infrared (FTIR) spectroscopy is a label-free, non-destructive analytical technique that can be used extensively to study a wide variety of different molecules in a range of different conditions. The aim of this review is to discuss and highlight the recent advances in the applications of ATR FTIR spectroscopic imaging to proteins. It briefly covers the basic principles of ATR FTIR spectroscopy and ATR FTIR spectroscopic imaging as well as their advantages to the study of proteins compared to other techniques and other forms of FTIR spectroscopy. It will then go on to examine the advances that have been made within the field over the last several years, particularly the use of ATR FTIR spectroscopy for the understanding and development of protein interaction with surfaces. Additionally, the growing potential of Surface Enhanced Infrared Spectroscopy (SEIRAS) within this area of applications will be discussed. The review includes the applications of ATR FTIR imaging to protein crystallisation and for high-throughput studies, highlighting the future potential of the technology within the field of protein structural studies and beyond.

1. Introduction

Our understanding of protein structure and function has advanced dramatically over the last 20 years or so, at least in part as a result of the time and effort spent on structural proteomic projects [1]. Obtaining high-resolution 3D protein structures is essential to gain insights into the mechanism of action of these important molecules, as well as to provide a potential framework for rational and targeted drug design. X-ray diffraction is the most commonly used method for determination of high resolution structures but obtaining well-diffracting protein crystals can be both difficult and time consuming [2]. As more and more structures are solved the remaining uncharacterised proteins tend to be those that are the most challenging to work with including multi-subunit complexes, unstructured proteins [3] and integral membrane proteins [4]. Methods to provide efficient high-throughput screening of conditions likely to yield diffracting protein crystals would greatly facilitate studies on the more challenging protein molecules. In addition although X-ray crystallographic studies can reveal much, such studies produce static snapshots of protein molecules and lack dynamic information key to understand the conformation changes associated with function.

Many of the most challenging types of proteins are prone to aggregation and unfolding as a result of the conditions used for isolation and final preparation [5]. Similar problems are known to occur in the case of biopharmaceuticals such as therapeutic antibodies and antibody fragments [6]. Whilst it is likely that increases in protein concentration can result in protein aggregation, the effects of the surfaces that proteins encounter during isolation are far less well understood [7,8]. A much better understanding of the effects of surfaces on protein behaviour has major implications for the development of biomedical materials, clinical diagnostics and cellular adhesion [9–11]. One prominent field is the study of the effect of surface properties on proteins which has major implications in the development of medical devices and implants [9,12]. The ability to understand the role that a particular surface has on a protein's conformation, in situ would be extremely valuable.

Infrared spectroscopy, in particular Attenuated Total Reflection (ATR) Fourier Transform Infrared (FTIR) spectroscopy, has significant potential as an analytical technique to facilitate both protein crystallisation studies and investigations on the effect of surface properties on protein behaviour as well as other key questions related to protein structure and function. This review focuses on the most recent advances in the field of ATR FTIR spectroscopy to proteins, particularly the development of ATR FTIR spectroscopic imaging and surface enhanced infrared absorption spectroscopy.

2. ATR FTIR spectroscopy

There are numerous reviews detailing infrared spectroscopy, therefore only the fundamentals will be discussed here. Infrared spectroscopy is the study of the interaction of infrared light with matter. When a beam of infrared light is directed at a sample, the wavelengths absorbed are dependent on the molecular vibrations of the substance. As such, based on the infrared absorbance of a sample one can determine both chemical and structural information from a sample. The multiple ways in which infrared light can be directed at samples and the range of

* Corresponding author. Tel.: +44 207 5945574.
E-mail address: s.kazarian@imperial.ac.uk (S.G. Kazarian).

1570-9639/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbapap.2013.07.015

detectors available allow for the analysis of a wide variety of samples in a range of conditions.

The most traditional method is transmission, where infrared light is passed through a sample sandwiched between two IR transparent windows. However, since the beam is travelling through the absorbing sample, this method requires short pathlengths of typically no more than 6–10 μm between the IR transparent windows, in the case of aqueous samples. This is particularly important when aqueous samples are to be studied because water is a strong absorber of IR light; too long a pathlength would result in all the IR radiation being absorbed by the sample and none reaching the detector. Reflectance infrared spectroscopy is another method where IR light is reflected off the external surface of a sample. It is similar to attenuated total reflection except the infrared beam is not directed through an infrared transparent medium to the sample.

ATR FTIR spectroscopy involves directing the infrared light at an interface between an infrared transparent material with a high refractive index called the internal reflection element (IRE, e.g. a prism made of ZnSe, diamond, silicon or germanium) and a sample on the surface of the IRE. The angle of incidence of the IR beam is greater than the critical angle and as such, total internal reflection occurs. At the reflecting surface, a standing wave of radiation called an evanescent wave is established and it is this evanescent wave that interacts with the sample, attenuating the infrared beam of light exiting the IRE. The depth to which the evanescent wave penetrates a sample is defined by the depth of penetration, the depth at which the evanescent wave is attenuated to 1/e, shown in Eq. (1). This is dependent on the angle of incidence and the refractive indices of both the prism and the sample. It is typically in the range of 0.5–2 μm although it should be noted that absorbance information is obtained from the sample beyond this range, as the evanescent wave probes deeper than the depth of penetration [13]. Since ATR FTIR is inherently a surface layer technique in that the evanescent wave only interacts with the several micrometer thick surface layer of the sample, it has advantages for protein studies in particular. In comparison with transmission, it is much more amenable to the study of aqueous samples in particular and the sample preparation is generally much simpler.

$$d_p = \frac{\lambda}{2\pi n_1 \left(\sin^2\theta - \left(\frac{n_2}{n_1}\right)^2\right)^{1/2}} \quad (1)$$

3. FTIR spectra of proteins — the amide bands

The use of IR spectroscopy to gain insight into protein structure and behaviour has been continually developed since the first work on characterising the spectral features of different polypeptide secondary structures and amino acids [14–16]. The infrared spectra of protein exhibit 5 characteristic bands, three of which are known as amide bands. These are most commonly used in infrared protein studies. The Amide I band, which has the strongest absorption of infrared light, is found between 1600 and 1700 cm^{-1}. It is primarily caused by stretching vibrations of C═O coupled weakly with C–N stretch and N–H bending. The exact band position is determined by the backbone conformation and the hydrogen bonding pattern within the protein molecule [17]. The Amide II band occurs at 1500–1600 cm^{-1} and is mainly derived from the C–N stretch along with N–H in-plane bending. Lastly, the Amide III band is found at 1200–1300 cm^{-1}. The vibrations responsible for this band are a complex mix of N–H bending and C–N stretching along with deformation vibrations of C–H and N–H [17–19].

Although all three bands can be useful to elucidate the secondary structure of a protein, the Amide I band is the most sensitive to structural changes and is the most commonly used in secondary structure analysis. Factors responsible for the conformational sensitivity of the Amide I band include hydrogen bonding and coupling between transition dipoles, both inter and intra molecular, which will impact the way in which a protein molecule interacts with IR light [20]. From the study of simple homopolypeptides of known single structure, the relationship between Amide I band position and structure has been proposed allowing predictions about the secondary structure of proteins to be made from IR spectra [16,18]. Further analysis of the Amide 1 band is required in order to obtain the types and amounts of secondary structure present; this is achieved through Fourier deconvolution and second derivative, curve fitting and spectral subtraction, for example. Multivariate analysis and other statistical methods are also increasingly being applied to the analysis of protein spectra because of their usefulness in interpreting the wealth and complexity of information contained within the spectra [21–23].

There are many examples in the literature of the assignment of protein secondary structure based on the analysis of the Amide I band [15–17,20,24]. In general, α-helical structures have a band peak at wavenumbers 1650–1658 cm^{-1}; β-sheet structures tend to have bands between 1620 and 1640 cm^{-1} and between 1670 and 1695 cm^{-1}; random coil structures occur at around 1644 cm^{-1} [20]. It is even possible to assign more complex structures such as an α-helix overlapped with random coil which can absorb IR radiation at 1654–1656 cm^{-1} [17]. Fig. 1 shows an example of a protein spectrum, the inset shows curve fitting of the Amide I band for different structural components [19].

The study of aqueous protein samples is challenging because of the overlap of the water bending mode with the Amide 1 band at 1600 cm^{-1}. This could be overcome through the use of D_2O; however this can affect the native secondary structure of the protein. Current FTIR spectrometers allow for accurate subtraction of the water band, although care should be taken to avoid over subtraction. There are several papers with guidelines for this procedure but it is widely accepted that the integrity of protein spectral data can be maintained by meeting two subtraction criteria; accurate subtraction of water vapour bands between 1800 and 1500 cm^{-1} and ensuring a straight baseline between 2000 and 1750 cm^{-1} upon water subtraction [24,25].

4. ATR FTIR spectroscopy of surfaces and proteins

The study of the interaction of proteins with different surfaces is important for the design of biomaterials, biomedical devices and for the production of biopharmaceuticals [10,26–28]. Both the surface chemistry and topography can play a major role in the behaviour of biological materials [29]. As described above, ATR FTIR spectroscopy is a surface layer technique where the absorbance is measured only from the portion of a sample in direct contact with the ATR surface rather than from the bulk. The surface of an IRE (or ATR crystal) can be easily

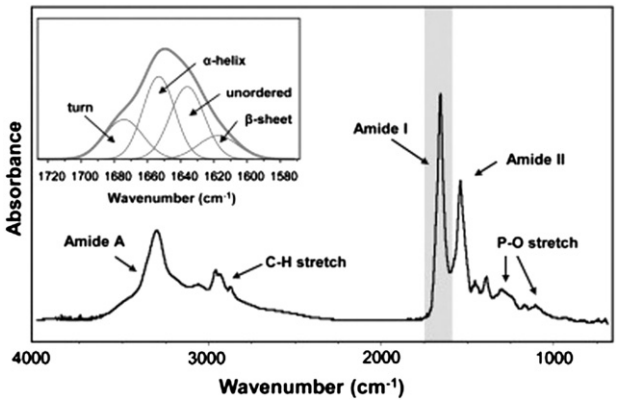

Fig. 1. FTIR spectrum of a typical protein illustrating the Amide I and Amide II bands at ~1650 cm^{-1} and ~1540 cm^{-1}, respectively. (Inset) Expanded view of the Amide I band, which can be deconvolved into its secondary structure components (Reprinted from R.W. Sarver and W.C. Krueger, *Protein secondary structure from Fourier transform infrared spectroscopy: A data base analysis*, Analytical Biochemistry 194 (1991) 89–100, with permission from Elsevier) [19].