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# Infrared Microspectroscopy study of insulin crystals at high pressure

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**Abstract.** During the last years the coupling of high pressure techniques and infrared spectroscopy has proven to be a very powerful tool in the study of conformational changes of proteins. Protein unfolding and monomerization are events that are expected to take place at high pressure due to the peculiarity of pressure to shift the system towards the state that occupies the minimum volume. We observed the growth of apparently cubic crystals at a pressure of about 4 kbar, subjecting to high pressure a solution of misfolded insulin. Even if high pressure is commonly used to tune the growth rate of crystals, protein crystallization at high pressure is not a well known process and no evidences of the particular case of insulin are present in literature.

## 1 Introduction

The amyloid diseases, which include Alzheimer's and Parkinson's diseases, the spongiform encephalopathies and type II diabetes, are characterized by the self-assembly and deposition of proteic material into insoluble ordered aggregates. So far, nearly 20 different amyloidogenic precursors have been identified although the detailed mechanism that leads to the formation of fibrils by these proteins is still unclear at a molecular level. For the difficulty in obtaining well diffracting crystals the atomic structure of fibrils has not yet been resolved. The accepted structural model for fibrils predicts the existence of a core protofilament composed of elements of  $\beta$ -sheets[1]. The sheets run parallel to the axis of the fibril and their component  $\beta$ -strands is thought to be perpendicular to this axis. The fibril is considered mature when the lateral packing between few extended protofilaments occurs. Insulin is a 51 residues protein central to regulate carbohydrate and fat metabolism in the body. It shows a high tendency to aggregate into amyloid-like fibrils *in vitro*, during its storage as pharmaceutical in the treatment of some forms of diabetes[2]. Due to the clinical relevance of insulin misfolding and its high tendency to aggregate into amyloid fibrils it is considered a model protein in the study of misfolding. When insulin is heated at high temperature and low pH, a series of structural changes occurs and results in the formation of the ordered aggregates[3]. High Hydrostatic Pressure has been proven to be a very powerful tool in the study of misfolding due to its effect on the energetic of the system that involves only variation on the volume contribution of Gibbs free energy[4]. High Pressure perturbations only affect the H-bonds of the system, tuning the conformation of the molecule towards the state that occupies the smallest volume. It causes the electrostriction of charged and polar groups, the elimination of packing defects, and the solvation of hydrophobic groups. Cavities and packing

defects are expected to be major contributors to volume changes and their presence will make the system more susceptible to pressure unfolding/dissociation. In the last years many studies remarked the high pressure ability to monomerize physiological assemblies and to unfold native. Moreover, the high pressure induced rupture of amyloid fibrils has been recently reported[5]. Since amyloids are temperature insensitive, pressure represents a unique tool for studying their thermodynamical stability. Infrared spectroscopy is a well established method for the analysis of protein secondary structure. The singular advantage of FTIR over other techniques is that spectra can be obtained for biological samples in a wide range of environments, and proteins can be studied in their physiological conditions. There is a wealth of information that can be used to derive structural information by analyzing the shape and position of bands in the Amide I region ( $1600\text{-}1700\text{ cm}^{-1}$ ) of the spectrum. The presence of a number of Amide I band frequencies have been correlated with the presence of  $\alpha$ -helical, antiparallel and parallel  $\beta$ -sheets and random coil structures[6]. In particular, it is now well accepted that the occurrence of fibrillation can be easily followed on infrared spectra from the observation of a very narrow component at about  $1620\text{ cm}^{-1}$ .

We studied the high pressure behavior of insulin fibrils in solution in the pressure range between 2.8 kbar and 11.4 kbar. During the compression cycle, we observed the formation of apparently cubic crystals. We present here the infrared microspectroscopy (IRMS) characterization of the high pressure crystals obtained. To determine their secondary structure, the crystals have been explored through the use of synchrotron light that allowed measurements on single crystals.

## 2 Experimental technique

### *Sample Preparation*

Insulin fibrils have been prepared following the head-induced BPI fibrillation protocol[3]. We dissolved 11mg of BPI (Bovine Pancreatic Insulin) powder in 1ml of  $\text{D}_2\text{O}$  at acidic pD ( $\sim 1.5$ ) (the pD has been adjusted adding DCI to the solution). The protein solution has been incubated at  $70^\circ\text{C}$  for 6 hours, then loaded into the diamond anvil cell.

### *HP-FTIR measurements*

The high pressure measurement has been performed through the use of a diamond anvil cell equipped with a screw mechanism to tune pressure. We used IIa diamonds with a culet diameter of  $600\text{ }\mu\text{m}$ , and a  $40\text{ }\mu\text{m}$  stainless steel 316L gasket, with a hole diameter of  $200\text{ }\mu\text{m}$ . The pressure range explored goes from 2.8 kbar to 11.4 kbar.

The absorption measurement has been done with a Hyperion IR microscope in the frequency range between  $1000$  and  $4000\text{ cm}^{-1}$ , at a resolution of  $4\text{ cm}^{-1}$  and 256 scans, using a Vertex Bruker interferometer coupled with the conventional Globar source and the MCT detector. The spatial distribution of the protein inside the pressure cell has been determined through the use of the FPA detector.

The single crystal absorption measurement has been performed coupling the infrared interferometer with the synchrotron source, the infrared microscope and the MCT detector. The beam size for synchrotron measurements on single crystals has been set to  $10\times 10\text{ }\mu\text{m}$ , to fit the dimension of crystals. We loaded into the cell, together with the sample, a small ruby sphere as pressure calibrant. Pressure calibration has been performed through the ruby fluorescence technique, using an home-made fluorescence system mounted on the infrared microscope.

## 3 Results

BPI (Bovine Pancreatic Insulin) fibrils have been subjected to a compression cycle, exploring a pressure range between 2.8 kbar and 11.4 kbar. In Figure 1 is reported the Amide I band of the whole sample during the compression cycle. Together with the high pressure spectra, of the sample loaded

into the diamond anvil cell, we reported the ambient pressure measurement, done using a standard liquid cell. The ambient pressure spectrum is composed of two  $\beta$ -extended peaks, at 1619 and 1630  $\text{cm}^{-1}$  and other components absorptions coming from intramolecular structures: 1647  $\text{cm}^{-1}$  (random coil), 1657  $\text{cm}^{-1}$  ( $\alpha$ -helix), 1663  $\text{cm}^{-1}$  ( $3^{10}$ -helix or sheets), 1678  $\text{cm}^{-1}$  (turns, loops and others). The presence of two peaks associated to the extended structure is due to the split of the lower frequency peak (1619  $\text{cm}^{-1}$ ) into two components. This phenomenon takes place when lateral packing between protofilaments occurs. We observed a very huge effect on the protein structure even at the lowest pressure applied (2.8 kbar). The components that can be found from the observation of the high pressure spectra are the following: 1619  $\text{cm}^{-1}$  ( $\beta$ -extended), 1636  $\text{cm}^{-1}$  (intramolecular  $\beta$ -sheet), 1652  $\text{cm}^{-1}$  ( $\alpha$ -helix), 1672  $\text{cm}^{-1}$  and 1684  $\text{cm}^{-1}$  (turns, loops and others).

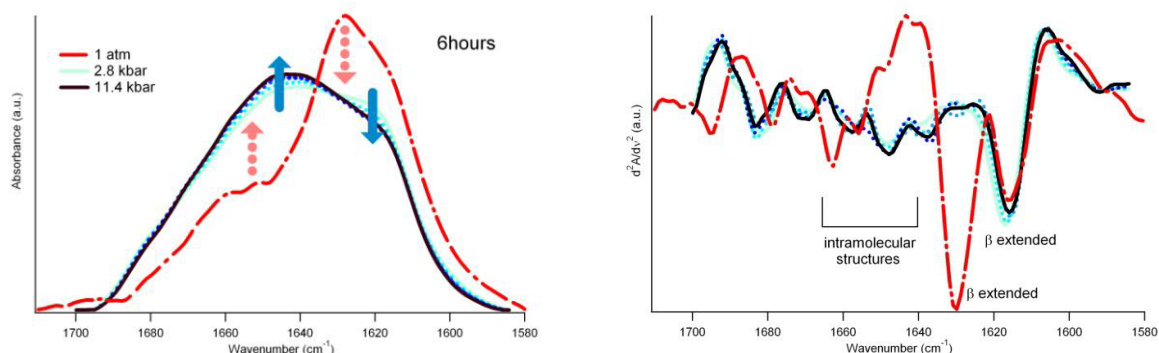


Figure 1: Absorbance (left) and 2<sup>nd</sup>-derivative (right) spectra of BPI fibrils at ambient pressure and during the compression cycle.

The presence of only one  $\beta$ -extended component at high pressure, remarked by the 2<sup>nd</sup>-derivative spectra, hints the absence of lateral packing between protofilaments, the solution is thus mainly populated by protofibrils. The Amide I area in the region of  $\beta$ -extended structures (1600-1640  $\text{cm}^{-1}$ ) significantly decreased during compression, sign of the induced rupture of part of the H-bonds composing the protofilaments. Conversely, the Amide I area above 1640  $\text{cm}^{-1}$ , where random coils and helices absorb, increased.

At a pressure of about 4 kbar, after about 1 hour from the pressure tuning, a small crystal appeared.

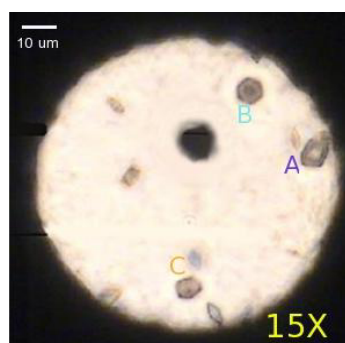


Figure 2: Picture of the sample after 4 days from the appearance of the first crystal (C). It is possible to recognize the pressure calibrant (black spot) in the upper part of the picture and ten small crystals all around it.

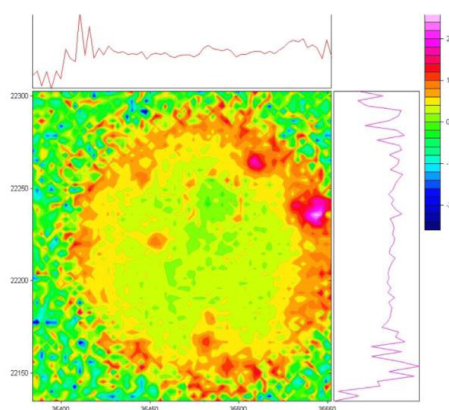


Figure 3: Amide I integrated signal of the sample after 4 days from the appearance of the first crystal. The image as been acquired through the use of the FPA detector.

The pressure has been kept constant to this value and after 4 days, about 10 crystals grew inside the cell (Figure 2). Crystals structure seemed to be apparently cubic. We acquired the FPA map of the whole sample surface to verify the spatial distribution of the protein inside the cell. The intensity distribution of the Amide I band inside the cell confirmed that the crystals were made of proteic matter (Figure 3).

To better characterize the secondary structure composition of crystals we also acquired single crystal absorbance (Figure 4). In Figure.5 are reported the Amide I bands of the three biggest crystals in the sample and of the soluble insulin acquired at 10x10 μm.

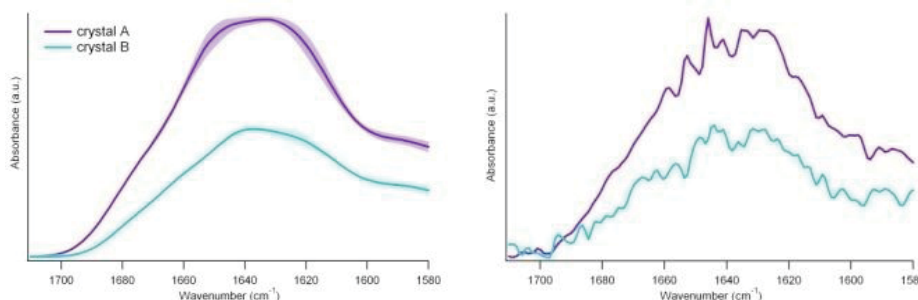


Figure 4 : Single crystal absorption spectra. (left) after 25-points smoothing filter and averaged on 5 measurements. The standard deviation is reported as shadow. (right) original spectra.

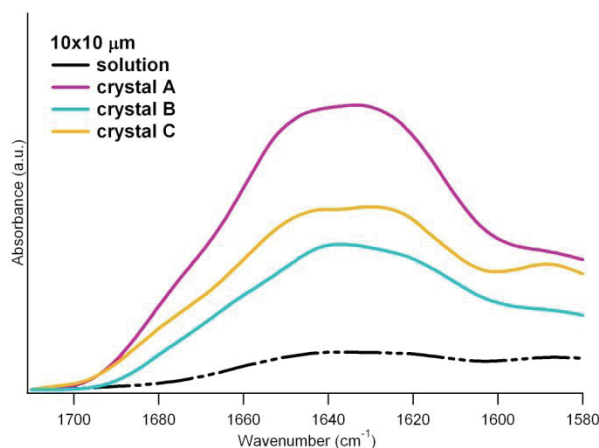


Figure 5 : Amide I band of the three biggest crystals inside the DAC. In dashed line is reported the absorbance of the solution around the crystals. Measurement performed with the synchrotron source and a beam size of 10x10μm.

For a more accurate evaluation of the secondary structure of the protein in crystalline form, it was necessary to consider also the contribution to the absorption coming from the optical path length occupied by the liquid sample. We approximated the shape of crystals to perfect cubes. To evaluate the true path length occupied by the soluble protein, we considered the difference between the thickness of the gasket, of 40 μm, previously calculated through the analysis of interference fringes, and the approximate height of the crystal, 10 μm, deduced from the observed lateral size of crystals. According to all these considerations, 3/4 of the total path length is occupied by the liquid sample, we thus subtracted the spectra following the equation:

$$Abs_{crystal} = 4 \times (Abs_{crystal-exp} - \frac{3}{4} Abs_{sol-exp})$$

Where  $Abs_{crystal-exp}$  and  $Abs_{sol-exp}$  are the signals acquired respectively on single crystal and on the solution at  $10 \times 10 \mu\text{m}$ , shown in Figure 5.

In Figure 6 are reported the single crystal Amide I bands after the subtraction procedure.

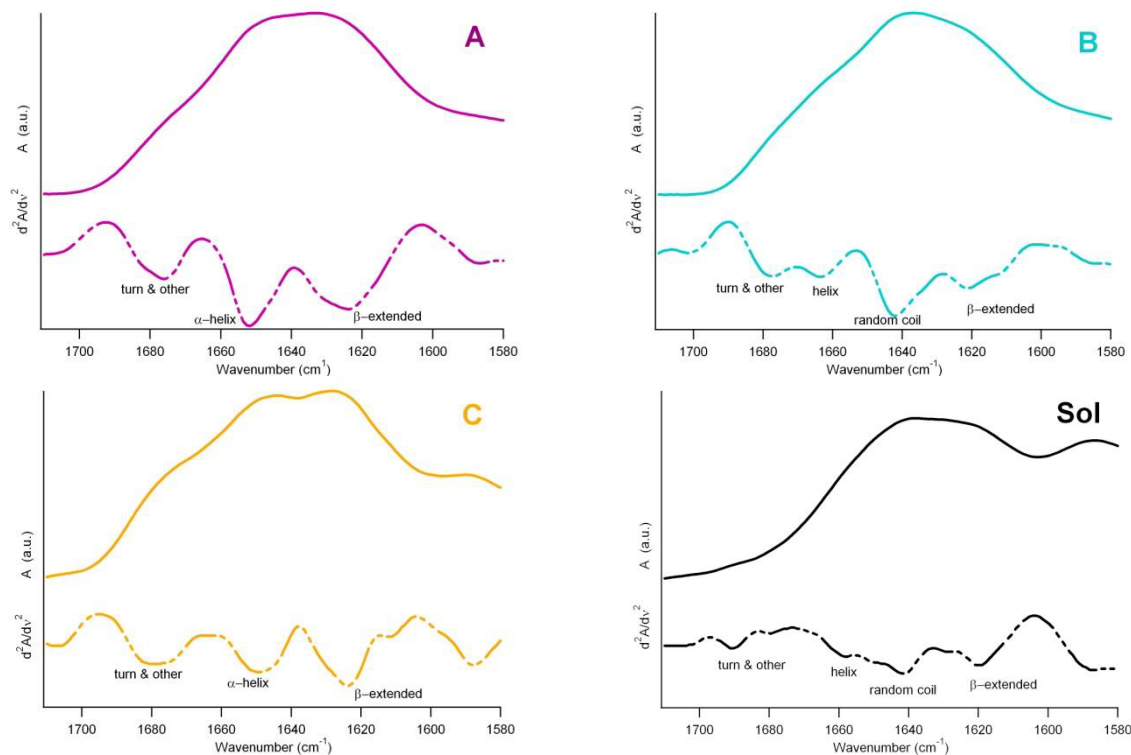


Figure 6: Normalized Amide I band of single crystals after the subtraction procedure. In black is reported the normalized Amide I band of the subtracted solution. The measurements have been performed with synchrotron beam size of  $10 \times 10 \mu\text{m}$ .

The subtracted single crystal bands are peaked around  $1640 \text{ cm}^{-1}$ , with three shoulders around  $1678 \text{ cm}^{-1}$ ,  $1646 \text{ cm}^{-1}$  and  $1622 \text{ cm}^{-1}$ . From the 2<sup>nd</sup>-derivative we found the presence of  $\beta$ -extended structures in all the three crystals measured, suggested by the minima occurring around  $1622 \text{ cm}^{-1}$  (Figure.6). Except for the crystal B, that possesses a component around  $1649 \text{ cm}^{-1}$ , corresponding to random coils absorption, the presence of a significant amount of  $\alpha$ -helices in the A and B crystals is evident by the huge minima around  $1656 \text{ cm}^{-1}$ .

The secondary structure of the soluble protein, evaluated by the observation of the spectrum of the solution at  $10 \times 10 \mu\text{m}$ , consists of all the absorptions found for crystals, with a very pronounced random coil component that makes it more similar to the crystal B.

We can therefore reasonably conclude that the crystalline form we obtained is composed of a partially folded intermediate.

The crystals have been compressed to 11 kbar then decompressed to 4 kbar. We found that they are stable in all the explored pressure range. After the pressure release the crystals slowly (about 4 minutes) disappeared. It is worth to notice that dimeric and hexameric insulin crystallizes at ambient pressure into cubic structures of similar dimensions[7].

## 4 Conclusions

The stabilization of insulin crystals under high pressure has been observed starting from a solution of fibrillated insulin and their secondary structure have been studied through the use of synchrotron radiation in the mid infrared range.

Based on the experimental findings, the following conclusions can be drawn:

1. A pressure of about 2.8 kbar can induce the rupture of the lateral packing between insulin amyloid-like protofilaments and the fragmentation of the fibrillar elongated structure.
2. A partially folded intermediate of insulin crystallizes at about 4 kbar and remains stable up to 11 kbar. The crystals have a size of about 10  $\mu\text{m}$  and are apparently cubic.

The question that arises from the present work concerns the hypothetical full reversibility of the insulin fibrillation process, at higher pressures, with the possible occurrence of the native protein refolding. Moreover, our experimental findings leave room for the eventual X-ray characterization of the crystallized structures. This could help to confirm data interpretation and eventually clarify the still unresolved atomic structure of amyloid-fibrils.

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