Wide-angle X-ray scattering as a probe for insulin denaturation

Wael M. Elshemey*, Ibtisam A. Mohammad, Anwar A. Elsayed

Biochemistry Department, Faculty of Science, Cairo University, Giza, Egypt

ARTICLE INFO

Article history:
Received 16 February 2010
Accepted 23 March 2010
Available online 31 March 2010

Keywords:
Insulin
WAXS
FTIR
Denaturation
Alpha helix

ABSTRACT

Wide-angle X-ray scattering (WAXS) from lyophilized protein is characterized by the presence of two relatively broad scattering peaks that are linked to protein structure. This work is concerned with the possibility of utilizing these peaks in the probing of the unfolding and breakdown of insulin. Native insulin is subject to thermal denaturation in the presence and in the absence of thiols catalysts. Denatured products are acid-trapped, lyophilized and monitored using WAXS in addition to Fourier transform infrared spectroscopy (FTIR), gel filtration chromatography and Transmission Electron Microscopy (TEM) as supportive techniques. Results show that the WAXS peak at a d-spacing about 10 Å is sensitive towards the α-helix content of insulin. A reduction in the intensity of such peak is proven to be directly linked to the reduction of native insulin having normal α-helix content. The supportive techniques confirmed the decrease in the α-helix content of insulin which accompanied the different denaturation treatments.

1. Introduction

There are several available techniques that can offer information about the conformation of protein [1–3]. Some of these are high resolution techniques that can provide a complete three dimensional structure of a given protein such as X-ray crystallography and nuclear magnetic resonance (NMR) [4–6]. Others can provide valuable information about protein secondary structure such as small angle X-ray scattering (SAXS) and circular dichorism (CD). Nevertheless, none of these techniques is perfect and there are always associated disadvantages and limitations.

X-ray crystallography requires high quality single crystals that are not always possible to obtain and the structural information retrieved from single static crystals would not always mimic the dynamic features of solvated protein [4]. NMR can provide information about protein in solution, yet it is limited to small proteins [7–10].

CD is limited to probing changes in the secondary structure of protein while SAXS is limited to probing structural alterations which are accompanied by changes in the radius of gyration [11].

Therefore, it is recommended that the study of protein structure is carried out using several structural techniques in order to build up a panoramic view of protein structure [4,1,12–17]. Moreover, many studies would only require limited information about protein conformation that can be preferably offered by a simple technique [4].

WAXS is one of the promising techniques that are simple and would help in the formation of an integrated image of the secondary, tertiary and quaternary structure of protein [11,18].

The WAXS profiles of dry [19,20], lyophilized [21] and solvated proteins [11,17,18] are characterized by the presence of two main scattering peaks. A scattering peak at d-spacing of about 4.5 Å that is attributed to the hydrogen bonding spacing of the alpha helix backbone in proteins with alpha helix as the main secondary structure element [22,23], or inter-strand hydrogen bonding spacing in proteins with beta sheet as the main secondary structure element [16,24,25]. This peak is characterized by being considerably broad with an observed stability against structural changes in protein. Another scattering peak exists at a d-spacing around 10 Å and is attributed to the inter-helix distance in proteins with alpha helix as the main secondary structure element [22,23], or inter-sheet spacing in proteins with beta sheet as the main secondary structure element [16,12,16,24–28]. It is a relatively narrow peak with a reported sensitivity towards protein secondary structure [23]. It has been reported that the intensity of such peak is correlated to the alpha helix packing and probably alpha helix content [29].

This work aims to evaluate the potential of WAXS as a probe for induced specific conformational changes in insulin. Complementary techniques such as FTIR, gel filtration chromatography and TEM are used in order to ensure the induction of specific structural changes in insulin and to offer supplementary means for proper understanding of WAXS results.
2. Materials and methods

2.1. Materials

Recombinant human insulin crystals are purchased from VACSERA, Egypt. The purity of the protein is greater than 98.8%. L-Cysteine hydrochloride (monohydrate) C3H8ClNO2S·H2O is purchased from Laba Chemie Ltd., India (molecular weight 175.64 g/mol, purity 99%). Urea CO(NH2)2 is purchased from Sigma (molecular weight is 60.06 g/mol, purity ≥ 99%). PBS Tablets (phosphate buffered saline) is purchased from bioshop Canada Inc. pH (one tablet in 10 ml) is 7.4 ± 0.1. Tris buffered saline tablets are purchased from Sigma (one tablet dissolved in 15 ml water gives 0.05 M NaCl). Trifluoroacetic acid (CF3COOH) is purchased from Sigma (purity 99%, density 1.53 g/ml at 25 °C and molecular weight 114.03 g/mol). Sephadex G-75 (particle size 40-120U, bed volume per gram dry gel: 12–15 ml) is purchased from Acros Organics, New Jersey, USA.

2.2. Sample preparation

2.2.1. Denaturation of the native insulin

2.2.1.1. Thermal denaturation. The native insulin is dissolved in 10 ml of the phosphate buffer (20 mM, pH 7.4, 100 mM NaCl) to give a solution of 0.05 g/10 ml. Denaturation is carried out at 50 and 70 °C for up to 6 h.

2.2.1.2. Thermal denaturation in the presence of thiols as a catalyst. The native insulin is dissolved in the phosphate buffer (20 mM, pH 7.4, 100 mM NaCl) to give a solution of 0.05 g/10 ml containing different concentrations of cysteine (Cys) (50, 100, 150 and 200 μM). Denaturation is carried out at 50 °C for up to 6 h.

2.2.1.3. Chemical denaturation in the presence of thiols as a catalyst. In the case of chemical denaturation, the native insulin (0.5 mg/ml) is incubated in the Tris–HCl buffer (0.1 M, pH 7.4) containing Cys (200 μM) and different concentrations of urea. Denaturation is carried out at 22 °C for up to 6 h.

In order to monitor the unfolding of insulin samples, different concentrations of urea are acid-trapped (quenched) with an equal volume of 4% aqueous trifluoroacetic acid (TFA) [30,31].

2.2.2. Lyophilization of protein samples

Control and treated insulin samples are freeze dried in order to remove the water content and obtain the powder form while preserving molecular conformation as possible. This is carried out using LABCONCO freeze drier, USA.

2.3. X-ray diffraction measurements

Control and treated lyophilized insulin samples are measured using the Philips X’pert Multipurpose X-ray diffraction system (MPD). The X-ray operating conditions are 40 kV and 40 mA. The device uses a Cu target (size 12 mm × 0.4 mm) to produce 8.047 keV collimated X-ray. Measurements are carried out in a step mode at a step equal to 0.5°. Diffraction data are collected using the PW 3011/10 proportional detector employing a graphite monochromator. The diameter of sample holder is of 25 mm and its depth is 5 mm.

2.4. Fourier-Transform infrared (FTIR) spectroscopy

Control and treated lyophilized insulin samples are measured using NICOLET 6700 FTIR Thermo scientific spectrometer, UK. The resultant spectra are smoothed with a seven-point Savitsky Golay smooth function to remove the noise [32–34]. After area normalization, the second-derivative spectra are obtained using the derivative function of Origin software Version 6.0. The difference spectra are obtained by subtracting the second-derivative spectra of different samples from control.

2.5. Gel filtration chromatography

A volume of 0.5 ml of control and treated insulin solution samples (0.05 g/10 ml) is fractionated using a Sephadex G-75 column (2 cm × 18 cm) equilibrated and eluted with PBS (pH 7.4). Protein fragments in collected fractions (1 ml each) are detected by measuring the absorbance at 280 nm using UNICO UV-2000 spectrophotometer, China.

2.6. Electron microscopy

Samples for electron microscopy are prepared for analysis on carbon-coated grids, negatively stained with 2% (wt/vol) uranyl acetate in water, samples are incubated for about half a minute on perforated carbon grids, and then analyzed using a JEOLEM.I230, Japan, transmission electron microscope operating at an accelerating voltage of 100 kV.

3. Results and discussion

3.1. WAXS from native and denatured insulin

Fig. 1 presents WAXS profiles of control insulin and insulin denatured at 50 and 70 °C for 6 h, respectively.

Fig. 1. WAXS profiles of control insulin and insulin denatured at 50 and 70 °C for 6 h, respectively.
Fig. 2. X-ray scattering peaks of control insulin and insulin denatured using different cysteine concentrations. The inset shows the whole measured region.

Fig. 3. WAXS profiles of control insulin and insulin denatured using different urea concentrations.

Fig. 2 shows the contribution of cysteine diffraction peaks to the whole WAXS profile.

Fig. 3 presents the region containing the two main protein scattering peaks (at 10 and 4.5 Å, respectively) of the WAXS profiles of control insulin and insulin denatured using different concentrations of urea in the presence of 200 μmole cysteine catalyst. The sharp diffraction peaks are due to the diffraction patterns of both cysteine and urea (see the inset of Fig. 3). Despite being too noisy due to contamination with the sharp diffraction peaks of cysteine and urea, one can still observe a dramatic decrease in the intensity of the scattering peak at 10 Å (1/d = 0.1 Å⁻¹) for denatured samples compared to control. The intensity of such peak is minimum for samples denatured using the highest concentrations of urea (5 and 6 M). Unlike the thermal denaturation using different thiol concentrations (Fig. 2), it is difficult to differentiate a gradual decrease in the intensity of 10 Å peak at different concentrations of urea [urea concentration as low as 0.5 M, has been reported to be able to dissociate hexameric insulin into dimmers [13,35]]. Nevertheless, the sharp decrease in the intensity of 10 Å peak still ensures the sensitivity of such peak towards the unfolding and breakdown of insulin in the presence of urea.

3.2. FTIR from native and denatured insulin

FTIR is a reliable technique for the determination of some main features of the secondary structure of protein in solution. The analysis of the amide I band (1700–1600 cm⁻¹) originating from the C=O stretching vibration of the peptide group, and the analysis of amide II band (1600–1500 cm⁻¹) due to N–H bending with contribution of C–N stretching vibrations would yield useful information about the conformation of protein [2,27,36].

In the present work, FTIR is employed in order to monitor the secondary structure of native and denatured insulin with possible correlation to the WAXS results.

Fig. 4 presents the amide region (from 1800 to 1400 cm⁻¹) of the area normalized FTIR spectra of native (control) insulin and thermally denatured insulin at 50 and 70 °C. The second-derivative spectra of native (control) insulin and thermally denatured insulin at 50 and 70 °C.

Fig. 5 shows the second-derivative spectra of native (control) insulin and thermally denatured insulin at 50 and 70 °C. The second-derivative spectra is a band narrowing technique which would help to enhance the resolution of the overlapping bands contributing to the amide I band [31,37,38].

The second-derivative spectra in Fig. 5 reveal that the reduction in the absorption of thermally denatured samples in the amide I region is due to the reduction in the amount of α-helices (as seen in the α-helix absorption region 1658–1648 cm⁻¹) [39]. This
is accompanied by an increase in the amount of high wave number \( \beta \)-sheets (1695–1680 cm\(^{-1}\)) and low wave number \( \beta \)-sheets (1640–1610 cm\(^{-1}\)) [40–42]. The extent of decrease in \( \alpha \)-helices and increase in \( \beta \)-sheets is presented by the difference spectra (second-derivative of denatured subtracted from control) in Fig. 5. The shaded columns relate corresponding regions of change in \( \alpha \)-helices and \( \beta \)-sheets content in the second-derivative and difference spectra.

The amide I and II regions of the area normalized FTIR spectra of native insulin and thermally denatured insulin in the presence of different concentrations of thiol (cysteine) catalyst are presented in Fig. 6. One can observe a decrease in the absorption at amide II band for all denatured samples compared to control. At the amide I band, there are apparent decrease in absorption for samples denatured using 100 and 150 mM cysteine and a gradual shift to higher wave numbers for the concentrations from 50 to 200 mM. This shift is probably due to a degree of transformation from \( \alpha \)-helix to \( \beta \)-sheet structure.

Further analysis using second-derivative and difference spectra (Fig. 7) shows that there exists a gradual decrease in the amount of \( \alpha \)-helix content with increase in cysteine concentration. This behavior is better observed in the \( \alpha \)-helix region of the difference spectra. Since this behavior matches the gradual decrease in intensity of the WAXS peak at 10 Å for the same conditions, one may understand this decrease in peak amplitude to be closely related to the decrease in the \( \alpha \)-helix content. A corresponding gradual increase in the high and low wave number \( \beta \)-sheet with increase in cysteine concentration is also observable in the \( \beta \)-sheet region of the difference spectra in Fig. 7.

The effect of different concentrations of urea catalyzed by 200 \( \mu \)M cysteine on insulin is also studied using FTIR spectroscopy (Fig. 8). A dramatic diminishing in amide II band is observed in all treated samples compared to control. This is accompanied by a corresponding increase in the absorption at 1450 cm\(^{-1}\). This behavior suggests that an exchange took place in the samples that is indicative of conformational change at the tertiary structure level [3,13]. The amide I band of the denatured samples exhibits two distinct absorption peaks characteristic of high and low wave number \( \beta \)-sheets instead of the absorption peak at 1650 cm\(^{-1}\) in case of control.

The second-derivative spectra and corresponding difference spectra (Fig. 9) illustrate clearly the large decrease in \( \alpha \)-helix content accompanied by corresponding pronounceable increase in high and low wave number \( \beta \)-sheets. The difference spectra of all urea concentrations show almost no considerable variations, a behavior comparable to WAXS results for the same conditions.

3.3. Gel filtration chromatography of native and denatured insulin

Fig. 10a presents the gel filtration chromatograms of control (native) insulin denatured using different concentrations of cysteine. The chromatograms reveal an apparent decrease in the amount of native insulin which is accompanied by the appearance of several low molecular weight fragments for all concentrations of cysteine and probably high molecular weight fragments in case of the 200 \( \mu \)M cysteine sample. The gradual reduction in the amount of native insulin with the increase in cysteine concentration is a result which apparently agrees with the WAXS and FTIR measurements. The appearance of low and high molecular weight species is probably due to the disulfide scrambling action of cysteine [30].
The denaturation of insulin using different concentrations of urea in the presence of 200 μM cysteine show a decrease in native insulin with increase in concentration of urea (Fig. 10b). This behavior is not so clearly distinguishable in case of WAXS and FTIR and would probably be attributed to the sensitivity of gel filtration chromatography towards molecular mass rather than protein conformation. At the right-hand side of the main elution peak there exist several low molecular weight products of denaturation, while at the left-hand side, there is a possible high molecular weight product (in case of 1 M urea) which seems to be out of the fractionation range of sphadex G-75 gel.

**Fig. 9.** The second-derivative (top) and difference (bottom) spectra of control insulin and insulin denatured using different concentrations of urea in the presence of 200 μM thiol catalyst.

**Fig. 10.** Gel filtration chromatograms of control insulin and insulin denatured using (a) different concentrations of cysteine (b) different concentrations of urea in the presence of 200 μM thiol catalyst.
3.4. Transmission electron microscopy of native and denatured insulin

Fig. 11a presents a micrograph of control (native) insulin. The micrograph shows no aggregation or fiber formation. After heating for 6 h at 70 °C, a net work of fibrils is evident (Fig. 11b). This observation is attributed to the fact that α-helical assemblies are triggered to unfold due to the thermal denaturation, they undergo conformational change into ordered β-sheet aggregates [43]. These aggregates have an elongated fibrous morphology and hydrogen bonding between chains in a β-conformation that extends along the long axis of the fiber. These fibers are known as amyloid fibrils [28].

The formation of amyloid fibrils after thermal denaturation is another evidence that supports the interpretation of WAXS and FTIR results which refer to a reduction in the α-helical content that is accompanied by an increase in β-sheets (responsible for the fibers observed in the TEM micrograph).

The formation of amyloid fibrils can be also observed for insulin thermally denatured (at 50 °C) in the presence of 200 μM cysteine (Fig. 11c) and for insulin denatured using 200 μM cysteine + 6M urea (Fig. 11d). In both cases the formation of fibrils is attributed to the rearrangement of β-sheets (which appear due to the unfolding and breakdown of insulin) into fibrous form. In other words, the presence of amyloid fibers in the TEM micrographs is an indication of the conformational change involving a transition from α-helix to β-sheet structure, a result which strongly agrees with the interpretations previously drawn in this study.

4. Conclusion

It has been shown that it is possible to monitor the unfolding and breakdown of insulin using WAXS technique. The scattering peak corresponding to a d-spacing of 10 Å has shown potential sensitivity towards the alpha helix content of insulin. The interpretations drawn from the WAXS technique are consistent with those revealed by FTIR, Gel chromatography and TEM techniques. The sensitivity of WAXS technique towards the alpha helix content of insulin is expected to be also useful in monitoring similar proteins with alpha helix as the main element of secondary structure. This would probably extend the range of application of the present study.

Acknowledgment

The authors would like to thank the Faculty of Science, Cairo University for supporting this work by the Graduate Research Challenge Fund (GRCF).

References