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Piotr Stefanowicz, Inga Petry-Podgorska, Karolina Kowalewska, Lukasz Jaremko, Mariusz Jaremko, et al.. Electrospray mass spectrometry as a method for studying the high pressure denaturation of proteins. Bioscience Reports, 2009, 30 (2), pp.91-99. 10.1042/BSR20090015 . hal-00479312

HAL Id: hal-00479312

<https://hal.science/hal-00479312>

Submitted on 30 Apr 2010

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Electrospray Mass Spectrometry as a Method for Studying the High Pressure Denaturation of Proteins

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Short title:

High Pressure Denaturation of Proteins

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THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20090015

Accepted Manuscript

Synopsis

High pressure denaturation of proteins can provide important information concerning their folding and function. These studies require expensive and complicated equipment. In this paper we present a new convenient method for studying high-pressure denaturation of proteins combining deuterium-hydrogen exchange and electrospray mass spectrometry. Application of various values of pressure causes different degrees of protein unfolding resulting in molecules with a different number of protons available for exchange with deuterons. After decompression a protein refolds and a certain number of deuterons is trapped within the hydrophobic core of a refolded protein. Redissolving the deuterated protein in an aqueous buffer initiates the D/H exchange of amides located on the protein surface only, which can be monitored under atmospheric pressure by mass spectrometry. Depending on the degree of deuteration after high pressure treatment, the D/H exchange kinetics are different and indicate how many deuterons were trapped in the protein after refolding. The dependence of this number on pressure gives information on the denaturation state of a protein. The distribution of deuterium along the sequence of a high- pressure-denatured protein was studied the electron capture dissociation (ECD) on a Fourier-transform mass spectrometer, enabling the monitoring of high pressure denaturation with single amino acid resolution.

Keywords:

electrospray, high pressure denaturation, hydrogen-deuterium exchange, proteins, secondary structure, ECD;

Introduction

The thermodynamic stability of proteins can provide important biological information concerning their folding and function. High pressure has been long known to change the protein's conformation and, therefore, alter the rate and the equilibrium constants of biological reactions. It exerts an influence on the properties of proteins by the rearrangement and/or destruction of noncovalent bonds, such as hydrogen bonds and hydrophobic and electrostatic interactions, which normally stabilize the tertiary structure of proteins [1]. Only the native and denaturated structures are well known. There are cases, however, when the intermediate state (a molten globule) is detectable [2]. It has been found that the residual secondary structure of the pressure-assisted, cold-denatured states is very similar to the structure of the early folding intermediates [3].

High pressure treatment of food or pharmaceutical agents (pascalization) is a new method of sterilization. Some reports suggest that high pressure is not only efficient for the inactivation of bacterial, viral, and fungal pathogens, but may also decrease the infectivity of prions [4]. There are evidences that high pressure can induce the reversible denaturation of some globular proteins [5-7]. Pressure is an ideal perturbant in the studies on folding equilibria as it gives valuable thermodynamic information without the need of altering the chemical composition of a solution. High-pressure techniques have been applied in the studies on protein folding and more recently, in the studies on the transition states of that process. Such studies require high-pressure techniques integrated with FTIR, NMR, small-angle X-ray scattering, densitometry, fluorescence, and 4th derivative UV absorbance spectra [8-12]. However, these methods need custom build instruments, specially designed for measurements under high pressure conditions. Such complicated and expensive equipment may limit the availability of those methods.

As the electrospray mass spectrometry (ESI-MS) has become an important tool for protein analysis due to its high specificity and sensitivity, [13] we decided to apply it in studies on high-pressure denaturation of proteins. A combination of isotopic exchange and mass spectrometry gives additional insight into the protein stability and conformation in a solution [13]. The hydrogen-deuterium exchange (HDX) is a technique that became one of the major experimental tools to probe both structural and dynamic features of proteins. The replacement of each labile hydrogen atom with deuterium (or vice versa) changes the mass of a protein by one Da.

Since deuteration increases the molecular mass of a protein, mass spectrometry is often used to monitor HDX. In each case the high sensitivity of a contemporary mass spectrometer allows monitoring subtle changes in a very small number of molecules therefore kinetic studies can be carried out. In contrast to other analytical methods, HDX-ESI-MS experiments can be performed successfully even on a mixture of proteins [14, 15]. Therefore, the method is very useful for a comprehensive study of compounds with different molecular masses which are difficult to separate by other techniques.

Herein we present a new convenient method for studying high-pressure denaturation of proteins by ESI-MS. We used the method to study the HDX in selected model proteins known to undergo a reversible high-pressure denaturation. As the electrospray mass spectrometric analysis can be performed only under atmospheric pressure, we developed a two-step approach for our purpose (Figure 1). The HDX rate in a protein is closely related to its local environment. In general, a protein in a tightly folded conformation has fewer protons available for deuteration, than the same unfolded protein. Application of various pressures causes different degrees of unfolding and consequently different amounts of protons are available for exchange with deuterons.

After decompression the protein refolds and a certain number of deuterons is trapped within the hydrophobic core of the refolded protein. Redissolving deuterated proteins in an aqueous buffer initiates the D/H exchange (DHX) of deuterons. The exchange rates of the peptide amides' deuterons located on a protein surface are in the range that can be monitored under atmospheric pressure by ESI-MS (Step II in Figure 1). Depending on the degree of deuteration after the first step, the DHX kinetics are different and reveal a number of deuterons trapped in the protein during its refolding. Although the use of the proposed method is limited to proteins that reversibly unfold under high pressure, it provides benefits of study of unpurified proteins or even complex mixtures of proteins, unlike other traditional approaches based on NMR, IR or fluorescence spectroscopy. Therefore, the proposed method may be very useful in the analysis of the folding and the unfolding of proteins in their natural environment.

The measurement of the molecular mass of a deuterated protein provides information on the total number of exchanged amide protons only. The localization of the incorporated deuterons may be achieved by enzymatic cleavage with pepsin followed by chromatographic separation and MS detection of obtained peptides [16]. Recently, another method of localization of deuterium atoms in a peptide molecule based on the fragmentation of a protein molecule by electron capture dissociation (ECD) was proposed and discussed [17]. Unlike collision induced dissociation (CID), [18] ECD proceeds with a relatively low degree of hydrogen scrambling, what makes it a method of choice for localization of deuterated amides within a peptide chain. A recent report suggests that this method may be extended to spatially resolved hydrogen exchange experiment on the intact molecule of a protein. This measurement was performed on a ubiquitin molecule by Konermann's group [19]. Herein we performed ECD experiments to reconstruct the distribution of deuterated and undeuterated amide protons along the protein sequence. This paper confirms Konermann's results and is the first one presenting the deuterium distribution within the whole sequence of a protein with a well defined conformation in a solution.

Materials and Methods

Reagents

Deuterium oxide (99.9%D), acetic acid (HPLC grade), formic acid (HPLC grade), acetonitrile (LC-MS grade), ammonium formate (LC-MS grade), hen lysozyme, bovine α -lactalbumin and ubiquitin were purchased from Aldrich.

High Pressure Equipment

The high-pressure denaturation experiments were carried out using a custom-made cylinder-piston type apparatus. The stoppered polypropylene syringe, containing the protein sample was placed in a high-pressure vessel filled with hexane as a transmission medium and compressed (0.1 MPa to 1400 MPa).

High Pressure Experiment Protocol I

The solutions of 1 mg of a protein dissolved in 1 ml of 10 mM ammonium formate in D₂O (pD 6.9) were incubated at given pressures from 0.1 MPa to 1400 MPa for 30 min at room temperature. The compression and decompression time was approximately 2 min. The samples were frozen in liquid nitrogen immediately after decompression and dried overnight in a freeze-dryer. The DHX experiment under atmospheric pressure was initiated by dissolving dried samples in a 10 mM solution of ammonium formate (pH 6.6) in water. The final concentration of a protein was 1 mg/ml. After a specified DHX time (2, 4, 8, 16, 30, 60, 120, 240, 360, and 480 min) 10 µl of a protein solution was taken and isotopic exchange was quenched with 10 µl of 5% acetic acid that was cooled to approximately 0 °C. The sample was immediately analyzed by ESI-MS. Protocol I was applied to lysozyme and α-lactalbumin.

High Pressure Experiment Protocol II

The solutions of 0.5 mg of a protein in 0.1 ml of 10 mM ammonium formate in D₂O were incubated at a given pressure (0.1 MPa to 1400 MPa) for 30 min at room temperature. After decompression samples were immediately diluted with 500 µl of 10 mM ammonium formate (pH 6.6) in water to initiate the DHX under atmospheric pressure. The final concentration of a protein was 0.5 mg/ml. The obtained solution was directly infused into the ion source. Protocol II was applied to lysozyme and ubiquitin.

ESI-MS measurement

The mass spectra were obtained on a Bruker MicroTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), equipped with Apollo II electrospray ionization source with ion funnel. The mass spectrometer was operated in the positive ion mode. Mass resolution was 15.000 FWHM. The instrumental parameters were as follows: scan range *m/z* 400–2300, dry gas - nitrogen, temperature 200 °C, reflector voltage 1300 V, detector voltage 1920 V. The sample in the solution described in *High Pressure Experiment Protocol I* and *II* was infused at a flow rate of 3 µL/min. Before each run the instrument was calibrated externally with the Tunemix™ mixture (Bruker Daltonics, Germany) in the quadratic regression mode. The mass accuracy for the calibration was better than 5 ppm. The ECD experiments were performed using an Apex-Qe 7T instrument (Bruker) equipped with the ESI source and a heated hollow cathode dispenser, using standard Bruker ECD settings. The cathode dispenser was heated gradually to 1.7 A. The ECD pulse length was set at 7 ms., and ECD bias was 0.7 V. Analysis and deconvolution of the obtained spectra were carried out with a Biotools (Bruker) software.

Calculation of the extent of deuterium incorporation

The deuterium content of deuterated proteins was determined by the difference in the average mass between deuterated and nondeuterated species.

$$D(r) = M_D - M_0$$

(where $D(r)$ is the number of remaining deuterons, M_D is the observed average molecular mass of a deuterated protein sample; M_0 is the observed average molecular mass of a non-deuterated protein sample). $D(r)$ was plotted (Figure 2) as a function of time and the data was fitted to the following equation:

$$D(r) = D_1 \exp(-k_1 t) + D_2 \exp(-k_2 t) + D_3$$

(where D_1 – deuterons exchanged with a fast kinetic constant k_1 , D_2 – deuterons exchanged with a lower kinetic constant k_2 , D_3 – deuterons fully protected from the exchange).

Mapping of the exchanged protons in the ubiquitin molecule

Ubiquitin incubated in D_2O under high pressure was diluted with the 100-fold excess of the ammonium formate (10 mM) aqueous solution. After 60 min of the back exchange, the solution of ubiquitin was brought to pH 3.5 with formic acid, diluted with acetonitrile (1:1) and the ECD spectrum was acquired immediately. The average molecular masses of the corresponding deuterated and undeuterated fragments of ubiquitin (M_{av}) were calculated using the formula:

$$M_{av} = \Sigma(M_i A_i) / \Sigma A_i$$

(where M_i are the molecular masses for particular isotopic peaks in the isotopic envelope and A_i are the abundances of corresponding isotopic peaks).

The number of deuterium atoms (D_n) in all of the identified fragments of the c_n and $(z+1)_n$ type were calculated from the difference in the average mass between nondeuterated and deuterated fragments of ubiquitin.

Results and discussion

A new method of studying high pressure protein denaturation by mass spectrometry was tested using three model proteins - lysozyme, α -lactalbumin, and ubiquitin - that unfold reversibly under high pressure. The CD spectra of those proteins dissolved in ammonium formate buffer measured before and after a high pressure (1400 MPa) treatment (HPT) are exactly the same (not shown in the experimental part). Additionally, the reversibility of high pressure denaturation of proteins is proven by a similarity of the charge state distribution of a protein before and after HPT [20].

To characterize the denaturation degree as a function of pressure, we denatured model proteins in a deuterated solvent (10 mM ammonium formate in deuterium oxide, pD 6.9) by HPT at room temperature. Then the high pressure was released causing the renaturation of a protein. We observed very low temperature changes (ΔT less than 2 °C) during the compression or decompression, suggesting that the thermal effect itself should not influence the protein's stability. The deuterated protein samples were freeze-dried and redissolved in the aqueous buffer at pH 6.6 (protocol I) or, alternatively, diluted 10 times with the aqueous buffer (protocol II). Monitoring of the protein's molecular weight changes enabled us to observe the dependence of the deuteration degree versus pressure (Figure 2). The mass spectrometry analysis of a protein incubated in the deuterated buffer under higher pressure reveals significant changes in the molecular mass of the studied proteins.

Both protocols provide similar conclusions on the tested proteins, although 10% of protein deuterons remain unexchanged in the case of protocol II. Figure 2.I.a presents an example of the D/H exchange kinetics of α -lactalbumin monitored by ESI-MS.

The high pressure experiment for this protein was performed according to protocol I. The time course of DHX depends on the HPT of the sample. The sample of the protein incubated under high pressure presents a higher content of deuterium (more than 60 deuterons for the sample incubated under pressure of 1400 MPa), than the protein incubated in the deuterated buffer at the atmospheric pressure (20 deuterons only). Deuterium entrapped inside the molecule of the protein is exchanged relatively slowly ($t_{1/2} \sim 10$ h at room temperature, pH 6.6). Changes in the content of deuterium plotted as a function of pressure provide

information on the denaturation of the protein under high pressure. The dependences of the number of deuterons trapped in the hydrophobic core of the proteins after the high pressure treatment, followed by the 1 hour backexchange in the aqueous buffer, as a function of the applied pressure (denaturation curve) are presented in Figures 2. b. The curves were fitted to the sigmoidal Boltzmann function (the general form of the equation is: $y = (A_1 - A_2) / [1 + \exp((x - x_0)/B)] + A_2$). A similar equation was used by Powell and Fitzgerald [21] to describe the HDX under denaturing conditions. For lysozyme data were analysed separately in two sets corresponding to the range 0.1-700 and 600-1400 MPa. Pressure below 400 MPa did not influence the content of deuterium in the protein sample. The renaturation of α -lactalbumin previously denaturated at the pressure of 600 MPa entraps approximately 20 deuterons more than the protein denaturated at 400 MPa. A further increase of pressure had very little effect on the number of additional deuterons trapped in the hydrophobic core of the protein. The transition midpoint of α -lactalbumin is close to 480 MPa. This value is in a good agreement with the data reported previously by Jonas [2]. The application of a combination of advanced high-resolution nuclear magnetic resonance techniques and high-pressure measurements, evidences that α -lactalbumin is completely unfolded under the pressure of 500 MPa, whereas it forms a molten globule state below 400 MPa.

The corresponding experiment for lysozyme was performed using both protocol I and II. In both cases the denaturation curves are similar and the denaturation occurs in two steps. The changes in the yield of ultraviolet fluorescence emission of lysozyme with pressure permitted the detection of two distinct steps of its denaturation, at 400-800 MPa and 800-1100 MPa (transition 1 and 2, respectively) [22].

Lysozyme contains 129 amino acid residues that fold into two structural domains: the α -domain consists of four α -helices and a 3_{10} helix; the β -domain includes a long triple-stranded β -sheet, a short double stranded β -sheet, a 3_{10} helix, and a long loop [23]. The dependence of a number of deuterons trapped within the protein molecule as a function of pressure suggests the existence of two stages of denaturation: the first in the range of 0.1-600 MPa and the second in the range of 600-1400 MPa. The presented plot (Figure 2.II.b and Figure 2.III.b) suggests a two-stage HPT unfolding of lysozyme that reflects transition stages 1 and 2, as suggested by Li [22]. Two transition midpoints were observed, at about 400 and 850 MPa, for the experiment performed according either to protocol I or II.

The pressure values causing appearance of transition stages 1 and 2 of lysozyme are in a good agreement for protocols both I and II. The ΔM_1 and ΔM_2 values (marked in Figure 2.II.b and Figure 2.III.b) represent the number of amide protons exchanged during the first and the second stage. The protons must be involved in the formation of the internal hydrogen bonds in the native structure and they are exposed toward the solvent in the transition stages 1 and 2. The ΔM_1 and ΔM_2 values are 11 and 42 respectively and the difference between them is 31. These results may lead to the conclusion that 11 amino acid residues in the protein interior are exposed to the solvent in the intermediate state, whereas additional 31 residues are exposed at a pressure higher than 1000 MPa. On the basis of the above results we can not point out the amino acid residues that undergo the isotopic exchange in the transition stages 1 and 2. However, Samarasinghe et al. [24] suggested that the α -domain undergoes denaturation at a lower pressure, whereas denaturation of the β -domain requires a higher pressure (above 500 MPa).

The pressure denaturation of ubiquitin was performed using protocol II. Ubiquitin shows a very simple denaturation curve with the midpoint at approx 500 MPa. (Figure 2.IV.b) The data obtained by Herberhold and Winter, [25] using high pressure FT-IR are in a good agreement with our results. The pressure dependence of the FT-IR spectra of deuterium-exchanged ubiquitin at pD 7.0 and 21 °C revealed drastic changes in the spectral shape of the amide I' band at 540 MPa [25]. The total number of protons exchanged during the

denaturation process is approx 20. Only one inflection point on the sigmoidal plot in Figure 2.IV.b suggests that unfolding of ubiquitin occurs in one cooperative step. This result is in an agreement with the results obtained by Jonas [3].

During HPT, the unfolded structure of ubiquitin exchanges all the amide protons present in the core of the protein fast. The incubation of ubiquitin in the aqueous buffer (performed after the renaturation caused by the decompression) results in a fast backexchange of the amide protons located in the unstructured fragments of the protein backbone. The deuterons which are involved in the formation of the hydrogen bonds stabilizing the α or β structures are protected from DHX.

We applied the ECD method to determine the distribution of deuterons along the sequence of ubiquitin. The molecule of ubiquitin is the most relevant model for such a study because the protein does not contain disulphide bonds, what makes ECD fragmentation clearer. Additionally, a moderate number of amino acid moieties makes the results obtained for ubiquitin easy to interpretate.

The solution of ubiquitin was exposed to the pressure of 800 MPa for 30 min. We observed that a further increase in pressure (up to 1400 MPa) did not influence the number of exchanged protons what indicates that a whole protein molecule is denaturated at 800 MPa. After the decompression, the solution of the protein was immediately diluted with the aqueous buffer (10 mM ammonium formate in H_2O) and incubated for over 60 min. That time was sufficient for the exchange of the majority of labile protons located on the protein surface. Finally, the sample was diluted twice with acetonitrile containing formic acid (0.4%) and immediately subjected to the ECD experiment. The obtained spectrum of ubiquitin reveals the presence of 18 deuterium atoms, which proves the retention of the majority of deuterons in the protein molecule at the experimental conditions. The ECD fragmentation was performed for the ion at m/z in the range of 714-718 which corresponds to deuterated protein molecules with the charge of +12. Figure 3 presents the crude fragmentation data.

The data obtained for the HPT protein were compared to those obtained for unmodified ubiquitin. The analysis of the ECD spectra was carried out with a Biotools program and is presented in the Table 1. Fragments detected in the ECD spectrum belong to class c_n (total number of identified c fragments is 37) and $(z+1)_n$ (19 identified fragments). The identified c_n and $(z+1)_n$ fragments cover 49% and 25%, respectively, of the ubiquitin sequence which allows us to map the distribution of deuterium atoms in the sequence of ubiquitin. Masses of deuterium containing fragments of the deuterated protein are shifted to higher m/z values as compared to the fragments of nondeuterated ubiquitin. The differences between these masses reflect the number of deuterons in the fragments.

Table 1 Identified c- and (z+1) - fragment ions of deuterated and nondeuterated ubiquitin obtained by ECD-MS. m/z corresponds to the average values calculated from the isotopic patterns of particular ions.

fragment ion	charge state	m/z nondeuterated	m/z deuterated	fragment ion	charge state	m/z nondeuterated	m/z deuterated
C4	1	537.59	539.82	C60	7	966.97	968.86
C5	1	636.70	639.05	C61	8	860.36	862.12
C7	2	433.49	435.01	C63	9	793.36	794.97
C8	1	979.15	982.52	C64	9	807.71	809.27
C10	1	1136.92	1140.43	C68	9	856.43	858.13
C11	2	633.23	635.03	C70	9	880.02	881.75
C16	2	912.08	914.97	C71	9	892.59	894.39
C17	2	961.66	964.57	C75	10	851.69	853.22
C20	2	1118.28	1121.13	(Z+1)8	2	434.54	434.95
C21	2	1175.86	1178.85	(Z+1)10	2	559.69	560.12
C23	2	1282.94	1286.23	(Z+1)11	2	610.24	610.85
C24	3	898.69	901.15	(Z+1)12	2	653.78	654.85

C27	3	1012.47	1015.18	(Z+1)13	2	718.36	719.27
C28	3	1036.20	1039.33	(Z+1)15	3	564.69	565.25
C31	4	869.76	872.27	(Z+1)16	3	602.40	602.90
C32	4	898.69	901.04	(Z+1)17	3	640.44	641.08
C33	4	930.58	933.05	(Z+1)18	3	694.83	695.72
C38	5	843.37	845.20	(Z+1)19	3	733.19	734.15
C39	5	866.40	868.42	(Z+1)23	3	885.70	886.62
C41	5	917.61	919.79	(Z+1)25	3	943.09	944.23
C44	6	828.34	830.52	(Z+1)30	5	677.38	678.07
C46	6	864.96	866.89	(Z+1)31	5	691.60	692.33
C47	6	874.50	876.44	(Z+1)49	7	791.21	792.18
C48	7	768.02	769.73	(Z+1)51	7	823.68	824.79
C50	7	802.51	804.25	(Z+1)52	8	735.11	736.17
C51	7	820.96	822.63	(Z+1)53	8	751.25	752.37
C57	8	797.18	798.82	(Z+1)72	10	803.94	805.18
C58	8	811.56	813.15	(Z+1)76	11	778.18	779.57
C59	8	950.66	952.49				

Figure 4 shows the content of deuterium D_n in the fragment ions generated upon ECD fragmentation. The number of deuterons for particular ions is plotted versus the residue number (n) separately for the c and (z+1) series. This plot shows regions of high and low slopes of the D_n number dependence on n. The high slopes correspond to the structured fragments. In contrast the low slopes correspond to the unstructured fragments of the protein chain characterized by a low deuterium content. The removal of consecutive non-deuterated amino acid residues from the low slope regions does not influence the number of deuterons trapped in the molecule. On the other hand, the removal of the amino acid residues containing deuterium in their amide bonds reduces the number of remaining deuterons and suggests that the given amide bond is involved in the formation of a hydrogen bond stabilizing the secondary structure of the protein molecule. Recently, the localization of deuterons situated within short peptides has been analyzed by Rand et al [17] by ECD. Our results show that the localization of deuterons by ECD may also be successfully performed on an intact deuterated protein.

The ECD data indicate that deuterium atoms remain in the ordered fragments of ubiquitin, mainly in β -strands 1-7 and 11-15 and in α -helical fragments. There is a difference between the deuterium content in the C- and the N- terminal part of that protein. The observed distribution of deuterium corresponds well to the structure of a protein molecule with a long and flexible C-terminal motif which is responsible for its interaction with other proteins.

Using the NMR technique, Pan and Briggs [26] and Bougault et al. [27] investigated the accessibility and the structural stability of residues along the ubiquitin polypeptide chain by monitoring the changes in the cross peaks' intensities in a series of 2D ^{15}N - ^1H HSQC experiments. The kinetic data concerning the exchange rate of particular protons measured by NMR are depicted in Figure 5. There is a good correlation between the data obtained by ECD and NMR. Both analyses reveal that the amide hydrogens from the unstructured regions undergo a fast isotopic exchange, whereas the hydrogens involved in the stabilization of β -strands and α -helices are exchanged significantly slower.

This correlation may further confirm that ubiquitin under the pressure of 800 MPa undergoes a complete denaturation and after decompression the protein refolds into its native state. Our results demonstrate that the ECD based procedure provides data that allow to evaluate the retention of deuterium in particular segments of the protein chain. This approach seems to be more convenient and faster as compared to the previous methods based on a fast peptic hydrolysis of a deuterated protein combined with the LC-MS detection of obtained fragments [16].

Conclusions

The application of the H/D exchange combined with ESI-MS methods for the analysis of a protein's high-pressure denaturation has been shown. Although the effects of high-pressure on a protein's stability and conformation were not analyzed directly, the proposed technique enables the observation of the two-stage unfolding process of lysozyme as well as the determination of the pressure values that totally unfold the studied proteins. The obtained values of critical pressure for the denaturation of all analyzed model proteins are consistent with those reported previously in literature, what confirms the usefulness of the proposed method. The deuteration pattern of a protein with a single amino acid resolution was established from the deuteration levels of the fragment ions generated by ECD. The results obtained suggest that a combination of the H/D exchange and ICR-electrospray mass spectrometry is a fast and reliable method of analysis of the reversibly denatured proteins.

Acknowledgement

This work was supported by a grant No. N N401 222734 from the Ministry of Science and Higher Education of Poland.

Figure captions

Figure 1 A schematic representation of the isotopic exchange of a protein under high pressure measured by ESI-MS. The first step is the incubation of a protein in the deuterated buffer at the defined pressure for 30 minutes. Under high pressure (P_2) more protons are exchanged with deuterons due to unfolding of the protein, as compared to the experiment at the atmospheric pressure (P_1). The decompression causes refolding of the protein and trapping a number of deuterons in its interior. The second step involves redissolving a deuterated protein in the aqueous buffer and ESI-MS monitoring of changes of the molecular mass with time.

Figure 2 High pressure denaturation of: **I:** α -lactalbumin (protocol I); **II:** lysozyme (protocol I); **III:** lysozyme (protocol II); **IV:** ubiquitin (protocol II).

a: DHX kinetics of proteins treated with D_2O at different pressures;

b: Graph of the number of deuterons trapped in the hydrophobic core of a protein after the high pressure treatment followed by the 1 hour backexchange in the aqueous buffer as a function of the applied pressure.

Legend: \square - 0.1 MPa, \bullet - 200 MPa, ∇ - 300 MPa, \blacktriangle - 400 MPa, \blacktriangleright - 450 MPa, \blacktriangleleft - 550 MPa, \blacksquare - 600 MPa, \circ - 800 MPa, \blacktriangledown - 1000 MPa, \triangle - 1200 MPa, \triangleright - 1400 MPa.

Figure 3 Crude ECD spectrum of the (+12) ubiquitin ion that allows sequencing of 60% of the protein chain. The spectrum is divided into two parts presented in panel A and B. The values of m/z and z for identified c- and (z+1)-ions are given in the Table 1.

Figure 4 Deuteration levels of the c- and (z+1)-fragment ions (line a and b, respectively) generated from ECD. The deuteration pattern is readily recognized from the deuterium levels of the fragment ions generated by ECD. Representation of the secondary structure motifs along the sequence is adapted from the ubiquitin NMR structure in the Protein Data Bank (PDB id 1d3z).

Figure 5 NMR H/D exchange data published previously by Bougault [27] for ubiquitin dissolved in phosphate buffer. Fast exchange protons are depicted by lower pascals. Protons with a low isotopic exchange rate (represented by higher pascals) are involved in the stabilization of the secondary structure of the protein. Representation of the secondary structure motifs along the sequence is adapted from the ubiquitin NMR structure in the Protein Data Bank (PDB id 1d3z).

The list of abbreviations

ECD	Electron capture dissociation
FTIR	Fourier transform infrared spectroscopy
NMR	Nuclear magnetic resonance
UV spectra	Ultraviolet spectra
ESI-MS	Electrospray mass spectrometry
HDX	Hydrogen – deuterium exchange
DHX	Deuterium – hydrogen exchange
CID	Collision induced dissociation
LC-MS	Liquid chromatography-mass spectrometry
HPLC	High performance liquid chromatography
CD	Circular dichroism
HPT	High pressure treatment
ICR	Ion cyclotron resonance

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