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ROLE OF ELECTROSTATICS IN THE THERMAL STABILITY OF UBIQUITIN
A combined DSC and MM study

M. F. M. Sciacca[1], *D. Milardi*[2], *M. Pappalardo*[1], *C. La Rosa*[1] *and D. M. Grasso*[1*]

[1]Dipartimento di Scienze Chimiche, Università di Catania, Viale Andrea Doria 6, 95125 Catania, Italy
[2]Istituto CNR di Biostrutture e Bioimmagini-Sezione di Catania, Viale Andrea Doria 6, 95125 Catania, Italy

The failure of the ubiquitin-proteasome system is involved in many diseases. Here, in order to assess the pH-induced changes in the physico-chemical properties of ubiquitin, DSC measurements have been carried out at $2.5<pH<7$. Parallel 'in silico' pH titrations, suggest that there is a direct relationship between the calorimetric data and the electrostatic properties of ubiquitin. It is suggested that these pH-induced changes in the properties of ubiquitin may play a role in determining its ability to properly conjugate with its physiological targets.

Keywords: *DSC, electrostatics, molecular modelling, ubiquitin*

Introduction

For most of proteins to be active, they need well-defined 3D structures alone or in complex. Folding is a process through which newly synthesized proteins get to the native state. When the native structure is compromised, the misfolded proteins are eliminated by the ubiquitin-proteasome degradation system [1]. Under certain circumstances, misfolded proteins escape the degradation process, leading the accumulation of protein aggregates such as amyloid fibrils [2]. A growing body of evidence indicates that the impairment of the ubiquitin-proteasome system, can be a contributing factor to many neurodegenerative disorders including Parkinson's, Alzheimer's, Huntington's and Prion diseases [1–6]. In particular, the sporadic forms of these diseases are believed to be linked to impaired equilibrium of environmental factors as pH, ionic strength and metals homeostasis in different cell compartments [7]. It is also known that the physiological pH value, in particular pathological conditions like inflammations or traumatic effects, is noticeably lowered down to 4–5 [8]. Moreover, not negligible amounts of ubiquitin have been found in the proteinaceous deposits observed in tissues of affected organisms [9, 10]. All these evidences are in accordance with the hypothesis that pathogenic chemical factors might compromise, inter alia, the stability and the correct functioning of ubiquitin and, on turn, the normal protein turnover leading to undesired protein accumulation. Consequently, it is important to establish which of these factors plays a major role in destabilizing ubiquitin. To this aim, in the past years a plenty of studies concerning the thermodynamic stability of ubiquitin as a function of pH, ionic strength and small molecules have been undertaken by DSC and chemical unfolding experiments [11–17]. In particular, it has been demonstrated that the contribution of charge-charge interactions between the solvent – exposed ionizable groups of ubiquitin are responsible for the high thermal stability of this protein at neutral pH [13]. Later on, the contribution of surface electrostatic charges to the stability of ubiquitin has been questioned using site-directed mutagenesis and specific chemical modifications to first replace all Arg residues with Lys, followed by carbonylation of Lys – amino groups [16]. According to the urea-induced unfolding data reported in that paper, the thermodynamic stability of charge-depleted ubiquitin is very similar, if not higher, to the wild type protein thus suggesting that charge-charge interactions are not responsible for the high thermodynamic stability of this protein. Although it is questionable if a great number of mutations leaves the global fold and/or the local native contacts of the protein unchanged, the apparent discrepancies between the two above mentioned set of experiments may be temptatively explained if we take into account that: *i*) urea-induced and thermally induced unfolding data are not directly comparable [18, 19], and *ii*) in determining the thermodynamic stability (ΔG), enthalpic factors are highly compensated by entropic factors related either to the protein (native or unfolded) or to the solvent. In order to address this issue, in the present work the effect of pH on the thermal stability of ubiquitin has been analysed in a range

* Author for correspondence: dgrasso@dipchi.unict.it

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of protein concentration and pH previously unexplored. In addition, a comparison of calorimetric parameters with the number of electrostatic charges of ubiquitin calculated as a function of pH by using the Delphi module of the insightII molecular modelling software has allowed us to find a direct relationship between T_m, ΔH, and the number of net charges of ubiquitin thus evidencing the determining role of the electrostatic interactions and of the topological distribution of charges in controlling the thermal stability of ubiquitin. The possible biological consequences of these results are briefly discussed.

Experimental

Materials

Chemicals

Ubiquitin from bovine red cells was purchased from Sigma Chemical Co. and purified by extensive dialysis against pure water for 24 h at a temperature of 4°C. The Spectrapor 5 membranes with a cut-off of 5000 Da were used. Purity was checked by comparing gel-electrophoresis runs of both purified and not purified samples. Gel-electrophoresis runs were carried out by dissolving the protein in a ‘sample’ buffer (50 mM tris-Cl pH 6.8, 5% (*v*/*v*) methanol, 10% (*v*/*v*) glycerol , 1% (*w*/*v*) SDS) and boiled for 5 min in order to allow the complete denaturation of the protein. The gel-electrophoresis runs were carried out in a 1× Running buffer (0.025 M tris, 0.192 M Gly, 0.1% SDS) for 2 h at 140 V.

Protein concentration in pure water was measured by UV absorbance using a molar extinction coefficient ε_{280}=1280 M^{-1} cm^{-1} [12]. Where necessary, the pH and the ionic strength of the protein solutions were adjusted by HCl and NaCl, respectively. Protein samples, after a preventive degassing process, were scanned at 0.5°C min^{-1} in the 20–120°C temperature range.

Methods

Differential scanning calorimetry

DSC runs were all performed on a VP-DSC instrument (MicroCal). All the experiments were performed alternatively at a protein concentration of 10 and 24 μM in a 0.5 mL cell. An extra external pressure of about 2 bar was applied on the solution. In order to obtain the excess heat capacity curves ($C_{p\ exc}$), the DSC curves, after the instrumental baseline correction, were subtracted from a baseline obtained by a third order polynomial fit of the pre- and post-transition cp trends. The reported data were the average of three experiments.

Molecular modelling

The native structure of ubiquitin was obtained from the Brookhaven Protein Databank (PDB code: 1ARR). Protons were added taking into account the ionization equilibria of the residues at pH 3, 5 and 7. The forcefield CVFF was used for all the calculations. The DelPhi module was used to calculate electrostatic distribution of charges around the protein surface at pH 3, 5 and 7, and adopting a dielectric constant value of 2 [20] for protein and 80 for water. All calculations were carried out using full Coulombic approximation. In particular, Poisson–Boltzmann solution for a single point charge q_i placed in the origin of the coordinate system has the Debye–Huckel form:

$$\phi_i = q_i \varepsilon_r^{-1} e^{-kr}$$

where ϕ represents the electrostatic potential (in units of kT/e assuming e as the electronic charge), ε is the dielectric constant of the solvent, k is the Debye–Huckel inverse length, and r is the spatial coordinates of the ith point. This equation was solved using finite approximation method [21–23]. The grid used for DelPhi calculation had a density of 69 points for Å^2.

Results and discussion

DSC curves at different pH and at protein concentrations of 24 and 10 μM are reported in the lower and upper panel of Fig. 1, respectively. A comparison between the two series of data obtained at 10 and 24 μM did not evidence remarkable differences, thus demonstrating that at these concentrations protein-protein interactions are negligible. Consequently, any effect on the thermal stability of ubiquitin in these experimental conditions has to be ascribed only to intramolecular interactions.

Figure 2 reports the values of T_m (panel a), ΔH (panel b), and ΔS (panel c), at a protein concentration of 10 μM (closed circles) and the net charge of the protein (open squares) as a function of pH. The number of net charges was obtained by calculating the number of protonated ionizable groups using the pH values recently reported for the solvent-exposed residues of ubiquitin [24]. It is shown that the thermal stability of the protein in terms of ΔH and T_m is not modified in the 7–4 pH range whilst it progressively decreases at lower pH values. Panel c) of Fig. 2 reports the ΔS calculated at the unfolding temperature and the net charge of the protein as a function of pH. It is evident that the ΔS of thermal unfolding decreases dramatically at increasing concentration of H^+ .

In order to better correlate the electrostatic effect on the thermostability of protein at different pH, we used the Delphi module in the InsightII molecular modelling software to obtain the topological distribu-

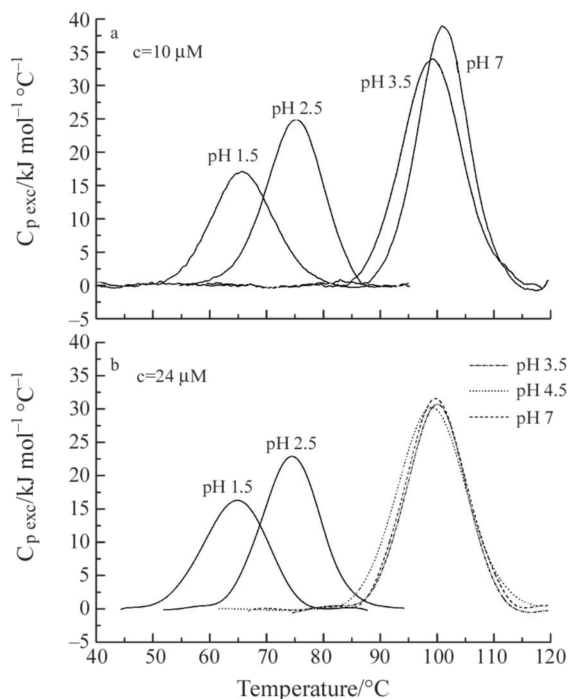

Fig. 1 DSC curves of ubiquitin at different pH and protein concentrations: a – 10 and b – 24 μM. Ionic strength and heating rate were kept fixed at 0.1 M and 0.5 K min^{-1}, respectively

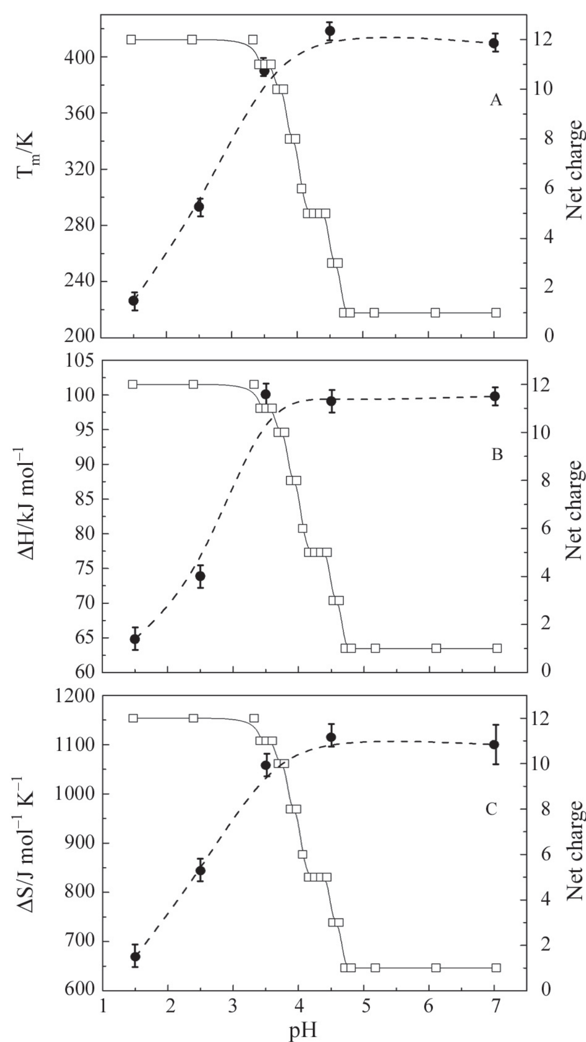

Fig. 2 Plots of unfolding T_m, ΔH and $\Delta S=\Delta H/T_m$ of ubiquitin at a protein concentration of 10 μM (panel A, B and C, respectively), reported as a function of pH (closed circles). In each panel, are reported in the right *Y*-axes (open squares) the number of net charges calculated as reported in the text

tion of charges on the surface of the protein. In Fig. 3 two pictures of the electrostatic distribution of the protein at pH 7 and 3 are reported. These pictures, in which the positive charges appear blue and the negative ones red, have evidenced that while at pH 7 in which the net charge of the protein is +1 attractions between opposite charges are possible, at pH 3 in which the net charge of the protein is +14 only the electrostatic repulsions are expected to occur. From the topological distribution of charges it is evident that besides the number of net charges, it should be also considered their topological distribution on the surface of the protein. In fact, in the pH 4.5–7 range the value of +1 for the net charge originates from 12 negative and 13 positive charges, in the pH 3–4.5 range the net value of +7 originates from 6 negative and 13 positive charges, while at pH lower than 3 the net value of +12 originates derive from one negative charge, the glicine, and 13 positive charges due to arginine, histidine and lysine residues.

The whole of the present data evidenced that the thermal stability of ubiquitin depends on the pH of the medium as a consequence of the modified distribution of electrostatic charges on the protein surface. In particular in the range 4.5<pH<7 the protein is stabilized by a network of attractive electrostatic interactions that maintain its structure ‘constrained’ as also confirmed by the high unfolding ΔS (about 1100 J K^{-1} mol^{-1}). This highly organized structure requires a very large energy (about 400 kJ mol^{-1}) and a high temperature (about 100°C) to be denatured. At pH<3 the repulsion among the positive charges destabilizes the protein. If compared with calorimetric parameters obtained at pH 7, at pH 2.5 T_m is lowered of about 26°, ΔH of 115 kJ mol^{-1}and ΔS of 260 J K^{-1} mol^{-1}. In the intermediate pH range the electrostatic repulsions are partially compensated by the attractive electrostatic forces between opposite charges. In the light of previous CD experiments showing that the stability of the secondary structure elements of ubiquitin does not depend on electrostatic charges [10], our results would suggest that the pH-induced reduction of protein stability should be related to its tertiary structure.

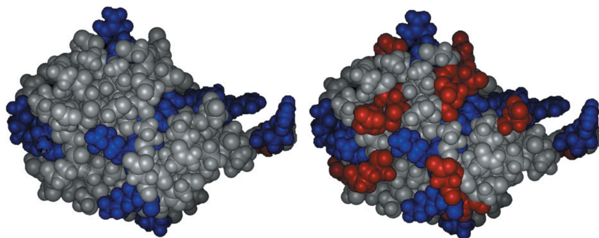

Fig. 3 Schematic representation of the distribution of electrostatic charges calculated at left – pH=3 and right – pH=7. Positive and negative charges are coloured in blue and in red, respectively

Conclusions

The role played by electrostatic interactions in ubiquitin stability evidenced here may have relevant biological implications. Degradation of a protein via the ubiquitin-proteasome pathway involves two discrete and successive steps: *i*) tagging of ubiquitin molecules to the substrate to synthesize the polyubiquitin chain proteolytic signal and *ii*) degradation of the tagged substrate by the 26S Proteasome complex [1]. Conjugation of ubiquitin to the protein substrate proceeds via a complex mechanism involving many ancillary proteins which finally leads to a covalent attachment of ubiquitin to the substrate. Many experimental evidences suggest that electrostatic forces play a key role in regulating the early steps of this process. In a recent paper [25] it has been observed that hHR23B, a protein known to participate in DNA repair, initially binds to ubiquitin through electrostatic interactions involving one domain whose surface map of electrostatic potential matches the ubiquitin counterpart. On the other hand, accumulating evidence suggests that extracellular and intracellular pH can change significantly under pathological conditions [26]. The local pH reductions from normal values could be even larger in some compartments as synaptic cleft and subsynaptic regions. Although aware that changes in the concentration of H^+ may be involved in many other biological disfunctions, still we believe that specific pH-induced modifications of the electrostatic properties of ubiquitin may be representative of a possible scenario in which an uncontrolled, pathological decrease of pH could be related to the impairment in the normal protein turnover in the cell.

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