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Elimination of the C-cap in ubiquitin—structure, dynamics and thermodynamic consequences☆

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Abstract

Single amino acid substitutions rarely produce substantial changes in protein structure. Here we show that substitution of the C-cap residue in the α-helix of ubiquitin with proline (34P variant) leads to dramatic structural changes. The resulting conformational perturbation extends over the last two turns of the α-helix and leads to enhanced flexibility for residues 27–37. Thermodynamic analysis of this ubiquitin variant using differential scanning calorimetry reveals that the thermal unfolding transition remains highly cooperative, exhibiting two-state behavior. Similarities with the wild type in the thermodynamic parameters (heat capacity change upon unfolding and *m*-value) of unfolding monitored by DSC and chemical denaturation suggests that the 34P variant has comparable buried surface area. The hydrophobic core of 34P variant is not packed as well as that of the wild type protein as manifested by a lower enthalpy of unfolding. The increased mobility of the polypeptide chain of this ubiquitin variant allows the transient opening of the hydrophobic core as evidenced by ANS binding. Taken together, these results suggest exceptional robustness of cooperativity in protein structures.

Keywords: Protein structure; Protein stability; Cooperativity of unfolding; Differential scanning calorimetry; Heteronuclear NMR; Circular dichroism spectroscopy

1. Introduction

Understanding the relationship between the linear amino acid sequence, the 3D structure and the thermodynamic stability of a protein still remains elusive. In an effort to elucidate the underlying principles it is particularly interesting to analyze cases in which a single amino acid substitution results in significant changes in the 3D-structure and/or stability.

Recently, the role of helix-capping interactions for protein structure and stability was explored by investigating the effects of amino acid substitutions at the C-cap position of the α-helix of ubiquitin. The single α-helix in ubiquitin comprises residues 24–33 and consists of three helical turns. The first residue proceeding the helix (C-cap residue) in the wild type ubiquitin is E34. Effects of amino acid substitution with all 20 naturally occurring amino acid at the C-cap on the stability of the ubiquitin variants revealed a number of interesting properties [1]. First, it was observed that stability changes correlate with the hydrophobic nature of residue 34. Second, far-UV CD and HSQC NMR experiments revealed no dramatic structural changes for all but one (Pro) amino acid substitution. Third, the Pro substitution in position 34 resulted in significant perturbations in the far-UV CD and HSQC NMR spectra, suggesting structural rearrangements in this ubiquitin variant. Here we report the results of the 3D structure determination of the 34P variant of ubiquitin using multidimensional

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heteronuclear NMR spectroscopy. In addition, the thermodynamic properties of the 34P variant were characterized by differential scanning calorimetry (DSC), chemical denaturation and fluorescence spectroscopy. A significant change in the protein structure is observed that involves conformational changes at the C-terminus of the α-helix of ubiquitin. The structural changes of this ubiquitin variant are accompanied by peculiar changes in the thermodynamic properties of the protein, possible sources of which are discussed.

2. Materials and methods

2.1. Protein mutagenesis, expression and purification

Mutations in the ubiquitin gene were introduced using QuickChange site directed mutagenesis kit. The presence of the desired mutations was confirmed by sequencing the entire gene. Proteins were expressed from plasmid carrying the ubiquitin gene under control of the T7 promoter in BL21(DE3) or JM109 (DE3) and purified as described previously [1]. Protein concentration was measured spectrophotometrically using a molar extinction coefficient of 1480 at 276 nm [1]. Correction for the light scattering was done as described [2].

2.2. Circular dichroism

CD spectra were measured at 25 °C on a Jasco J-715 spectrophotometer as described elsewhere [1]. Each spectrum was the result of averaging five individual spectra using 0.1 cm cylindrical quartz cell. The protein concentration in all cases was 0.4 mg/ml in 30 mM glycine pH 3.5. Measured values of the ellipticity, Θ, were converted into the ellipticity per amino acid residue base, [3] as:

$$[\Theta] = \frac{\Theta \cdot \mathrm{MW_R}}{l \cdot c} \quad (1)$$

where l is the optical length of the cell, c is the concentration of the protein, and $\mathrm{MW_R}$ is the average mass of the amino acid residues taken to be 114 Da.

Urea-induced unfolding experiments were performed in 5 mM glycine/HCl, pH 3.5 by monitoring the changes in ellipticity at 222 nm. Changes in urea concentration in solution were achieved using an automated titration system based on a Microlab 500 dispenser (Hamilton, Reno, NV) as described [4]. Analysis of the data was done according to the linear extrapolation model using nonlinear regression routines as described elsewhere [5].

2.3. Differential scanning calorimetry

DSC experiments were preformed on a VP-DSC (MicroCal, Northampton, MA) instrument at a scan rate of 90°/h. All experiments were carried out in 30 mM glycine or sodium acetate buffers. The protein concentration in the DSC experiments varied between 1.5 and 3.5 mg/ml. All temperature-induced unfolding transitions were reversible as judged by the area under the excess heat capacity function of first and second scans. Calorimetric profiles were analyzed according to a two-state transition model using the nonlinear regression routine NLREG and in-house written scripts [6].

2.4. Fluorescence spectroscopy

Steady-state fluorescence experiments were performed on a FluoroMax Spectrofluorimeter with DM3000F software (SPEX Industries, Inc.) as described [7,8]. A constant temperature in the thermostated cell holder (25 °C) was maintained using a circulating water bath. A quartz cell with a 1 cm path length was used. The buffer used in all titration experiments and in measurements of fluorescence emission spectra was 25 mM sodium acetate, pH 5.5. Concentration of the stock solution of ANS (8-anilino-1-naphtalene sulfonic acid) in buffer was determined spectrophotometrically using an extinction coefficient of 5000 M^{-1} cm^{-1} at 355 nm [9]. Stock solutions of protein and ANS were mixed to the final concentrations 10 μM and 25 μM, respectively. All experiments were run in triplicate, intensity was corrected for dilution, and average values are reported. The excitation wavelength used was 350 nm.

2.5. NMR data acquisition

Isotopically labeled proteins (^{15}N and $^{15}N/^{13}C$) were prepared using MOPS based minimal media with ^{15}N-ammonium chloride and ^{13}C-glucose as the only source of nitrogen and carbon, respectively [1,2,10]. Ubiquitin samples for NMR experiments were prepared by dissolving the lyophilized protein samples in 5% acetic acid with subsequent dialysis against 30 mM acetate buffer, pH 5.0. NMR experiments were carried out on samples containing 1–2 mM of suitably labeled protein. All spectra were recorded at 25 °C on Bruker DMX600 or DMX500 instruments equipped with a triple-resonance, triple-axes gradient probe. Backbone assignments of the protein were based on ^{1}H-^{15}N-HSQC, 3D-HNCACB, CBCA(CO)NH, HNCA, HNCO, and HBHA(CO)NH spectra [11] and H(CCO)NH-TOCSY and C(CO)NH-TOCSY experiments [11–15] were used for side-chain assignments NOE assignments were made using a 3D-^{15}N NOESY-HSQC [16] and a 3D NN-NOESY-TROSY-HMQC experiments. Residual dipolar coupling data were obtained for ^{15}N and ^{15}N,^{13}C labeled protein samples using two alignment media. The first medium contained 5% of dodeca-alkyl-penta(ethylene glycol) and hexanol, with a molar ratio of the glycol derivative to hexanol of 0.96 [17]. The second alignment medium was a 6% polyacrylamide gel (5.4 mm stretched in a 4.1-mm internal diameter NMR tube) containing 36% w/v acrylamide, 0.92% w/v *N*,*N*′-methylene bisacrylamide (ratio 39:1) [18]. One-bond ^{15}N–$^{1}H(N)$ and ^{15}N–$^{13}C'$ and two-bond $^{13}C'$–$^{1}H(N)$ dipolar couplings were measured using sensitivity-enhanced E-COSY-type HSQC experiments [19]. One-bond $^{13}C_\alpha$–$^{13}C'$ and $^{1}H_\alpha$–$^{13}C_\alpha$ dipolar couplings were measured using 3D-$^{13}C'$-coupled/^{1}H-decoupled HNCO and 3D ^{1}H-coupled HCA(CO)N experiments, respectively. Identical samples without the alignment media were used to collect reference