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Folding thermodynamics of c-Myb DNA-binding domain in correlation with its α-helical contents

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ABSTRACT

The conformational and thermal stabilities of the minimum functional unit for c-Myb DNA-binding domain, tandem repeat 2 and 3 (R2R3), were analyzed under different pH conditions, ranging from 4.0 to 7.5, using circular dichroism and differential scanning calorimetry. Secondary structure analysis showed that the solution pH largely affects the conformational stability of the protein domain. Of all conditions analyzed, the α-helical content was maximal at pH 6.5, and the thermal stability was highest at pH 5.0. Thermodynamic parameters for thermal unfolding of R2R3 were determined using differential scanning calorimetry, and the origin of folding thermodynamics at the different pHs and its correlation with the α-helical content were further analyzed. It should be noted that the α-helical content correlates well with the enthalpy change in the pH range from 4.5 to 7.5, suggesting that the strength of hydrogen bonds and salt bridges needed for maintenance of helical structure is related to enthalpy in the native state. Under physiological pH conditions, c-Myb R2R3 exists in the enthalpically unstable but entropically stable state. Due to loss of rigid structure and high stability, the protein can now obtain structural flexibility, befitting its function.

1. Introduction

Tertiary structures of proteins are maintained by intramolecular interactions including covalent and non-covalent interactions such as disulfide bonds, salt bridges, hydrogen bonds, and hydrophobic interactions. Owing to protein architecture, some proteins, such as protease inhibitors, are rigid and highly stable, while others such as enzymes fluctuate largely in solution. In other words, there exists a close correlation between the protein stabilities and its functions. "Rigidness" and "softness" are usually balanced in every protein in addition to its individual domains. These properties are regulated by intramolecular interactions, especially by non-covalent bonds, which can be subtly or drastically altered by environmental factors. Non-covalent interactions can be influenced by physical perturbation like temperature and pressure, and chemical perturbations such as pH, ionic strength, denaturant, and buffer components. The ionization state of residues is one of the important factors responsible for maintenance of the protein tertiary structure and is gravely affected by solvent conditions such as pH and ionic strength [1–5]. Structural and physical properties of model peptides which form typical α-helix and/or β-sheet structures, under different pH conditions have been reported previously [6–8]. In most of these analyses, the protein stability was analyzed only with respect to Gibbs free energy change (ΔG). Precise folding thermodynamics including enthalpy (ΔH) and entropy changes (ΔS) in correlation with tertiary structure can provide critical information on protein folding, including the strength of intramolecular interactions and the protein flexibility. In this study, we used the functional unit of protein with a helix-turn-helix motif, to analyze the pH-dependent changes on tertiary structure and folding thermodynamics.

The *c-myb* protooncogene product (c-Myb), is a transcriptional factor, with three functional domains involved in DNA-binding, transcriptional activation, and negative regulation [9]. The DNA-binding domain consists of three imperfect tandem repeats, denoted as R1, R2, and R3 at the N-terminus. Each repeat has a very similar folding architecture containing three well-defined helices [10,11]. Portions of second and third helices form the helix-turn-helix motif. The last two repeats, R2 and R3, are necessary for the specific binding to DNA containing consensus sequence, P_yAACNG, where P_y indicates a pyrimidine. The R1 is not essential for the

Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; TFE, trifluoroethanol; T_m, transition temperature; T_d, denaturation temperature; ΔC_p, heat capacity change; ΔG, Gibbs free energy change; ΔH_{cal}, calorimetric enthalpy change; ΔH, enthalpy change; ΔH_{vH}, van't Hoff enthalpy change; ΔS, entropy change.

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specific DNA recognition of c-Myb, and is considered to enhance the stability of R2R3 complex with DNA [12]. The thermal stability of R2 is lower than that of R1 and R3, because only R2 has a large internal cavity in the hydrophobic core [11,13]. Val103 is located in the hydrophobic core of R2 and participates in the large internal cavity, and mutation of Val103 affects the stability and the function [14–16]. Previous experiments have shown that perturbations in temperature and pressure lead to major fluctuations in the c-Myb DNA binding domain, especially in R2, under physiological conditions [17,18]. The mutation analysis of c-Myb DNA-binding domain also supports the notion that unique conformational fluctuations are critical for DNA-binding function [15,18–20]. Using the functional unit of c-Myb DNA-binding domain, R2R3, we found clear difference in secondary structure and thermal stability with pH ranging from 4.0 to 7.5. This unique property of R2R3, which enables large fluctuations, makes it possible to analyze the correlation of helical content with folding thermodynamics precisely with greater precision.

2. Materials and methods

2.1. Protein expression and purification

R2R3* was over-expressed in *Escherichia coli* and purified. The expression and purification methods were described previously [15,20]. Briefly, the overexpressed proteins in *E. coli* BL21 (DE3) were purified by using ammonium sulfate precipitates, phosphocellulose (P11, Whatman), and gel filtration (Sephacryl S-100, GE healthcare) columns. The purified proteins were concentrated and the buffer was exchanged to 20 mM sodium-acetate buffer (pH range from 4.0 to 5.5) or 20 mM potassium-phosphate buffer (pH range from 6.0 to 7.5) using Amicon Ultra-4 (Millipore). The purity of each protein was determined to be more than 95% by SDS/PAGE analysis. The protein concentrations were determined from UV absorption at 280 nm and were calculated by using the molar absorption coefficient of $3.7 \times 10^4\ M^{-1}\ cm^{-1}$ [21]. Prior to the current analyses, the Cys130 in R2, which is the only cysteine residue in the c-Myb R2R3 and is located at a position equivalent to an isoleucine in R3, was replaced with Ile, to facilitate protein purification and characterization. It has been shown that the affinity and the specificity of mutant C130I are similar to those of the wild type [21,22]. Therefore, the C130I protein was used as the standard R2R3, which is denoted as R2R3*.

2.2. Circular dichroism (CD)

Far-UV (200–250 nm) and near-UV CD (250–340 nm) spectra were recorded on a Jasco J-820 spectropolarimeter at 20 °C equipped with Peltier-type temperature control system, as described previously [20]. The spectra of R2R3* were obtained in 20 mM sodium acetate (pH range from 4.0 to 5.5) or potassium phosphate (pH range from 6.0 to 7.5) buffer, using quartz cell with 0.2 cm path-length. The protein concentrations were 0.1 mg ml^{-1} and 1.0 mg ml^{-1} for far- and near-UV respectively. CD spectra were obtained using scanning speed of 20 nm min^{-1}, a time response of 1 s, a bandwidth of 1 nm, and an average over 4 or 8 scans, for the far- and near-UV range, respectively. The α-helical contents, f_H were calculated as reported previously [23].

$$f_H/\% = \frac{-([\theta]_{222} + 2340)}{30300 \times 100} \tag{1}$$

The melting curves were recorded in temperature mode at 222 nm, from 20 to 80 °C with a heating rate of 1.0 °C min^{-1}. The protein concentrations were 0.02 mg ml^{-1} and the quartz cell with 1 cm path-length was used. The analysis of the transition curves obtained by temperature-scanning CD measurements was performed on the basis of two-state transition model, as described previously [16,20].

The molar ellipticity data of temperature-scanning CD (Y) were fitted with the weighted-mean combination of two linear functions for the folded and the unfolded states, that is,

$$y_n = A_n(T - T_m) + B_n \tag{2}$$

and

$$y_d = A_d(T - T_m) + B_d \tag{3}$$

from Eqs. (2) and (3), as shown below:

$$Y = y_n(1 - f_D) + y_d f_D \tag{4}$$

Here, f_D represents the molar fraction of the unfolded state and be expressed with the function of ΔG_{01}, the free energy of the transition:

$$f_D = \frac{\exp(-\Delta G_{01}/RT)}{\{1 + \exp(-\Delta G_{01}/RT)\}} \tag{5}$$

ΔG_{01} is also the function of the temperature involving three parameter, which are heat capacity change (ΔC_p), transition temperature (T_m), and van't Hoff enthalpy change (ΔH_{vH}):

$$\Delta G_{01} = \Delta C_p T \ln\left(\frac{T}{T_m}\right) + \left\{\Delta C_p - \left(\frac{\Delta H_{vH}}{T_m}\right)\right\}(T - T_m) \tag{6}$$

where ΔC_p determined in the DSC experiments was used. Substituting Eqs. (5) and (6) into (4), Y results in the function containing six parameters, that is A_n, A_d, B_n, B_d, T_m and ΔH_{vH}, to be determined. The curve fitting was carried out by the non-linear least-square method on Origin 5.0 software.

2.3. Differential scanning calorimetry (DSC)

DSC experiments were carried out on a Nano-DSC calorimeter (TA instruments), as described previously [20]. The data were collected by heating the solution from 5 to 90 °C at a rate of 1 °C min^{-1} and the sample concentrations were 2.0 mg ml^{-1} in 20 mM sodium acetate (pH range from 4.0 to 5.5) or potassium phosphate (pH range from 6.0 to 7.5) buffer. The outer buffer solution recovered from final exchanged experiment was used in the reference cell for each case. The data were analyzed using CpCalc software supplied by the manufacture, in order to subtract the baseline from heat capacity data and convert to molar heat capacity. The fitting analysis to obtain the denaturation temperature (T_d), heat capacity change (ΔC_p), and calorimetric enthalpy change (ΔH_{cal}) were performed by the least-squares methods on Origin 5.0 software. The ΔC_p was calculated from the difference in the C_p values between unfolded and folded states at T_d. ΔH_{cal} was calculated by integrating the area in each heat capacity curve. The ΔH_{vH} was calculated by the next equation for assuming the two-state transition.

$$\Delta H_{vH}(T_d) = 4RT_d^2\left\{\frac{C_p(T_d)}{\Delta H_{cal}(T_d)}\right\} \tag{7}$$

where R is the gas constant. Using the ΔC_p values with the ΔH_{cal} and T_d values, the thermodynamic parameters of unfolding as a function of temperature, $\Delta G(T)$, $\Delta H(T)$, and $\Delta S(T)$, could be calculated from the following equation, on the assumption that the ΔC_p is constant within the temperature range analyzed, as described previously [20,24].

$$\Delta G(T) = \Delta H_{cal} T_d\left\{1 - \left(\frac{T}{T_d}\right)\right\} - \Delta C_p T \ln\left(\frac{T}{T_d}\right) - \Delta C_p(T_d - T) \tag{8}$$

$$\Delta H(T) = \Delta H_{cal}(T_d) + \Delta C_p(T - T_d) \tag{9}$$