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Thermodynamic effects of multiple protein conformations on stability and DNA binding

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ABSTRACT

The side-chain conformations of amino acids in the hydrophobic core are important for protein folding and function. A previous NMR study has shown that a mutant protein of transcriptional activator c-Myb, I155L/I181L R3, has multiple conformations and increased fluctuation in comparison with the wild type. To elucidate the quantitative correlation of structural fluctuation with stability and function, we analyzed the thermodynamic effects of I155L and I181L mutations, using R2R3 that encompasses the minimum specific DNA-binding region. Circular dichroism and differential scanning calorimetry measurements showed that the mutation of I155L had little effect on stability, while the I181L mutation significantly destabilized the protein. It is noteworthy that the decreased stability resulting from the I181L mutation was mainly due to decreased enthalpy change, which is partially compensated by decreased entropy change. Isothermal titration calorimetry measurements showed that the specific DNA-binding affinity was decreased owing to the I181L mutation, which was due to decreased binding entropy change. Entropy in the folded state, which corresponds to the DNA-free state, increases due to the I181L mutation because of the increased conformational fluctuation observed in I155L/I181L mutant of R2R3 by CLEANEX-PM NMR analysis, which in turn results in decreased folding entropy and DNA-binding entropy changes.

Introduction

Proteins fluctuate in solution, and this dynamic behavior is critical for protein function [1–3]. Although protein structures have been determined at atomic resolution, most of them are stable structures that can be crystallized, and the averaged NMR structures that can be influenced by the major population among the various conformations in solution. In order to understand the real view of a protein and its function, the dynamic behavior should be added to the X-ray or NMR structure. A protein in the native state has multiple conformations in solution in general, including the most stable and low-lying excited ones, both of which are in thermodynamic equilibrium, although the minor populated conformation is difficult to detect. The degree of protein fluctuation among the multiple conformations can be determined by thermodynamic analysis as the conformational entropy. The protein fluctuation can also be analyzed by using NMR methods such as spin relaxation and $^{1}H/^{2}H$-exchange experiments.

The side-chain conformations of amino acids in the hydrophobic core are important for protein folding and function. Transcriptional activator c-Myb specifically binds to DNA, and its DNA-binding domain consists of three imperfect 51- or 52-residue repeats [4]. The last two repeats, R2 and R3, are sufficient for the recognition of the specific DNA sequence containing PyAAC($^{G}/_{T}$)G, where Py indicates a pyrimidine [5]. Each repeat has three α-helices that form a helix-turn-helix motif, which contributes to DNA binding and is maintained by the hydrophobic core including three conserved Trp residues (Fig. 1) [6]. Three Ile residues, namely, Ile-155, Ile-169, and Ile-181, are also involved in the hydrophobic core of R3 [7]. A previous NMR study has shown that a mutant protein of c-Myb R3, I155L/I181L, has multiple conformations, attributed to increased side-chain conformational entropy due to the mutation of Ile to Leu [8]. The two distinguishable conformations detected by NMR made it possible to analyze the effect of protein dynamic behavior on structure and function, especially in the view of conformational entropy. In the NMR analysis of R3 Ile mutant proteins, the unique structure of the I155L mutant could be determined, showing that the overall structure is similar to that of the wild type, while that of the I155L/I181L mutant could not, because many minor peaks appeared in the two-dimensional $^{1}H–^{15}N$ heteronuclear multiple quantum coherence spectroscopy spectra, due to the multiple conformations [8]. The lower protection factors of the I155L/I181L mutant determined by the NMR $^{1}H/^{2}H$-exchange experiments supported the notion that protein fluctuation, at least between the two conformations, would be increased upon the

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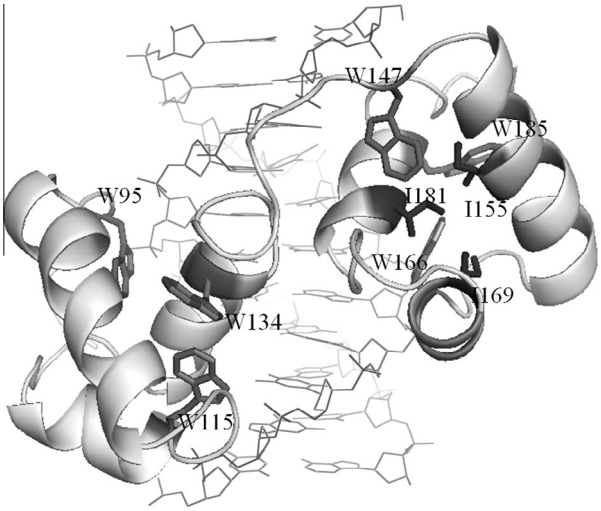

Fig. 1. The DNA-complexed structure of the c-Myb DNA-binding domain (PDB code, 1MSE). The side-chains of Trp-95, Trp-115, Trp-134, Trp-147, Trp-166, Trp-185, Ile-155, Ile-169, and Ile-181 are indicated by thick lines with the one-letter amino acid codes.

I181L mutation [8]. Although the previous study showed the effects of conformational entropy only qualitatively, it could not identify the quantitative effects on the total free-energy balance on the protein stability. In addition, since R3 alone was used, it was still unclear whether the mutation affected the function, i.e., DNA binding.

In this study, we analyzed the thermodynamic effects of multiple conformations on stability and DNA-binding function using the I155L/I181L R2R3 mutant and its control proteins. Considering that conformational entropy increases upon the I181L mutation, the ΔS value of folding thermodynamics, F (folded state) ↔ U (unfolded state), becomes smaller, and that of binding thermodynamics, protein (DNA-free state) + DNA ↔ complex (DNA-bound state), becomes smaller, assuming that the conformational entropies in the unfolded and the DNA-bound states are unchanged upon the mutation. The increased conformational fluctuation upon the I181L mutation in R2R3 at neutral pH could be observed by the Phase-Modulated CLEAN chemical EXchange (CLEANEX-PM[1]) NMR experiments, which detects $^{1}H^{N}$ exchange rates with the solvent ^{1}H [9]. Prior to the current mutational 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 [10,11]. Therefore, the C130I protein was used as the standard R2R3, to which additional mutations such as I155L and I181L were added. In this study, the C130I protein is denoted as R2R3*, and C130I/I155L and C130I/I155L/I181L are simply denoted as I155L and I155L/I181L.

Materials and methods

Preparation of proteins and DNA

The expression and purification methods of R2R3* and its mutants were slightly modified from the previous method [12]. Following the step of phosphocellulose column (P11, Whatman), the proteins were further purified using a gel filtration column (Sephacryl S-100, GE healthcare) connected to an AKTA prime plus system. The purified proteins were concentrated and the buffer was changed to PBS (pH 7.4) using Amicon Ultra-4 (Millipore). For the NMR experiment, the ^{15}N-uniformly labeled R2R3*and I155L/I181L were over-expressed in *Escherichia coli* in the M9 minimum medium containing 1.0 g/L $^{15}NH_4Cl$ and 4.0 g/L glucose. The protein concentrations were determined from UV absorption at 280 nm and were calculated by using the molar absorption coefficient of $3.7 \times 10^4\ M^{-1}\ cm^{-1}$ [11], determined by the amino acid composition analysis.

The 22-mer oligonucleotide, CACCCTAACTGACACACATTCT, was synthesized by Operon Biotechnologies, Inc., and purified by high performance liquid chromatography with a C_{18} reversed-phase column. The purity was determined to be about 95%. The complementary strands were annealed in PBS, generating the cognate DNA, MBS-I. The DNA concentrations were determined from UV absorption at 260 nm using the molar absorption coefficient of $1.3 \times 10^4\ M\ basepair^{-1}\ cm^{-1}$.

Circular dichroism (CD) measurements

Far-UV (200–250 nm) and near-UV (250–320 nm) CD spectra were recorded on a Jasco J-820 spectropolarimeter at 20 °C equipped with Peltier-type temperature control system. The spectra were obtained in PBS (pH 7.4), using quartz cell with 1.0 cm path-length. The protein concentrations were 0.02 mg/ml and 0.2 mg/ml for far- and near-UV respectively. CD spectra were obtained using scanning speed of 20 nm/min, 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 ranges, respectively. The melting curves were recorded in temperature mode at 222 nm, from 20 to 85 °C with a heating rate of 1.0 °C/min. 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 [13]. The molar ellipticity data of temperature-scanning CD (Y) were fitted with the weighted-mean combination of the two linear functions for the folded and the unfolded states, that is, $y_n = A_n(T - T_m) + B_n$ and $y_d = A_d(T - T_m) + B_d$, as shown below:

$$Y = y_n(1 - f_D) + y_d f_D \quad (1)$$

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

$$f_D = \exp(-\Delta G_{01}/RT)/\{1 + \exp(-\Delta G_{01}/RT)\} \quad (2)$$

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

$$\Delta G_{01} = -\Delta C_p T \ln(T/T_m) + \{\Delta C_p - (\Delta H_{vH}/T_m)\}(T - T_m) \quad (3)$$

Substituting Eqs. (2) and (3) into (1), Y results in the function containing seven parameters, that is, A_n, A_d, B_n, B_d, ΔC_p, T_m, and ΔH_{vH}, to be determined. The curve fitting was carried out by the non-linear least-squares method on Origin 5.0 software.

Differential scanning calorimetry (DSC) measurements

DSC experiments were carried out on a Nano-DSC II calorimeter (TA instruments). The data were collected by heating the solution from 5 to 80 °C at a rate of 1 °C/min. The sample was reheated without exchanging the solution in the cells to check the reversibility. The outer buffer solution recovered from final dialysis experiment was used in the reference cell for each case. The protein concentrations were 0.5 mg/ml in PBS buffer (pH 7.4). The data

[1] *Abbreviations used:* CLEANEX-PM, Phase-Modulated CLEAN chemical EXchange; CD, circular dichroism; ΔC_p, heat capacity change; T_m, transition temperature; ΔH_{vH}, van't Hoff enthalpy change; DSC, differential scanning calorimetry; T_d, denaturation temperature; ΔH_{cal}, calorimetric enthalpy change; τ_m, mixing time in CLEANEX-PM experiments; ITC, isothermal titration calorimetry; MBS-I, DNA fragment containing the Myb-binding site in the simian virus 40 enhancer sequence; n, stoichiometry of binding; K_a, binding constant.