Biochemistry and Biophysics Reports 11 (2017) 99–104

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Unveiling the folding mechanism of the Bromodomains

Maria Petrosino[a,1], Daniela Bonetti[a,1], Alessandra Pasquo[b], Laura Lori[a], Roberta Chiaraluce[a], Valerio Consalvi[a], Carlo Travaglini-Allocatelli[a,*]

[a] Dipartimento di Scienze Biochimiche "A. Rossi Fanelli", Università di Roma "Sapienza", P.le A. Moro 5, 00185 Rome, Italy
[b] SSPT-BIOAG-BIOTEC ENEA Casaccia ENEA, Rome, Italy

ARTICLE INFO

Keywords:
Bromodomain
Protein folding
Folding intermediate

ABSTRACT

Bromodomains (BRDs) are small protein domains often present in large multidomain proteins involved in transcriptional regulation in eukaryotic cells. They currently represent valuable targets for the development of inhibitors of aberrant transcriptional processes in a variety of human diseases. Here we report urea-induced equilibrium unfolding experiments monitored by circular dichroism (CD) and fluorescence on two structurally similar BRDs: BRD2(2) and BRD4(1), showing that BRD4(1) is more stable than BRD2(2). Moreover, we report a description of their kinetic folding mechanism, as obtained by careful analysis of stopped-flow and temperature-jump data. The presence of a high energy intermediate for both proteins, suggested by the non-linear dependence of the folding rate on denaturant concentration in the millisec time regime, has been experimentally observed by temperature-jump experiments. Quantitative global analysis of all the rate constants obtained over a wide range of urea concentrations, allowed us to propose a common, three-state, folding mechanism for these two BRDs. Interestingly, the intermediate of BRD4(1) appears to be more stable and structurally native-like than that populated by BRD2(2). Our results underscore the role played by structural topology and sequence in determining and tuning the folding mechanism.

1. Introduction

Proteins involved in the regulation of histone post-translational modifications, such as those involved in acetylation, phosphorylation or methylation, play a pivotal role in the control of gene expression [1], therefore acting as "proof-editors" of the genetic code. Proteins acting on the histone acetylation processes can be grouped in at least three different subsets, on the basis of their specific function: "writer" proteins (as Histone AcetylTransferases, HATs) responsible for the addition of acetyl groups to specific lysine residues, "eraser" proteins removing specific acetyl groups (as Histone DeAcetylases, HDACs), and "reader" proteins, endowed with the ability to recognize and bind to specific histone acetylated lysines (AcK), such as the Bromodomains (BRDs) [2,3].

BRDs are conserved structural motifs of about 100 amino acids that are often present in large multidomain proteins involved in a variety of cellular processes, such as chromatin remodeling, post-translational modifications or transcriptional control [4]. Members of the BET (Bromo-Extra-Terminal domain) family (comprising human BRD2, BRD3, BRD4 and BRDT) display common modular architecture with two highly conserved amino-terminal BRDs, and a less conserved C-terminal recruitment domain. Mutations or chromosomal rearrangements affecting BRDs have been linked to various human diseases including cancer, and therefore BRDs are currently considered a promising target for the development of small-molecule inhibitors aiming at interfering with aberrant transcriptional processes in such diseases [5–7].

The structure of a variety of BRDs has been solved and shows a conserved left-handed helical bundle composed by four α-helices termed αZ, αA, αB and αC (from the N-terminal to the C-terminal helix) connected by loop regions of variable length (ZA and BC loops) (Fig. 1). From a structural point of view the BRDs therefore belong to the all-α fold class. Structural analyses of acetylated peptide-BRD complexes have shown that the AcK binding site is a hydrophobic cavity present on top of the helical bundle whose surface is shaped mainly by the ZA and BC loops. Not surprisingly, given the pivotal role played by BRDs in a variety of patho-physiological processes, a growing number of studies are currently focusing on their ligand binding affinity and specificity. However, little is known about the dynamic properties of these domains and, to our knowledge, no information is available about the mechanisms of folding of BRDs. This lack of information is somewhat surprising as it is known that for many proteins a relationship exists between

* Corresponding author.
E-mail address: carlo.travaglini@uniroma1.it (C. Travaglini-Allocatelli).
[1] M.P and D.B. contributed equally for this work.

http://dx.doi.org/10.1016/j.bbrep.2017.06.009
Received 29 March 2017; Received in revised form 23 June 2017; Accepted 28 June 2017
Available online 08 July 2017

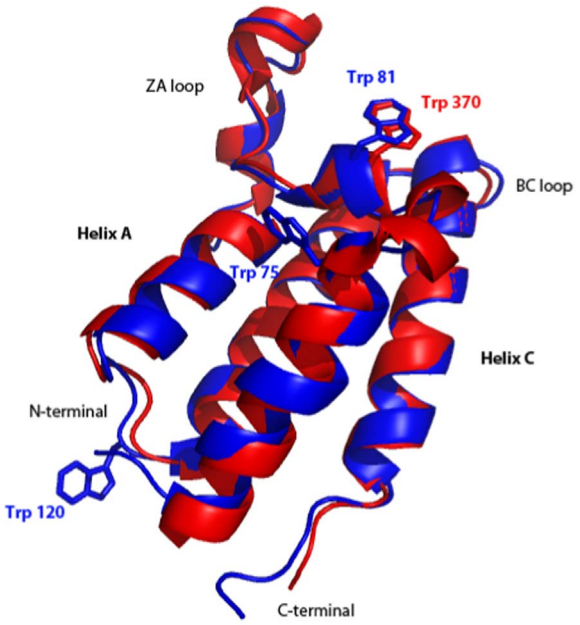

Fig. 1. Structural alignment of BRD2(2) (pdb id: 3oni), shown in red, and BRD4(1) (pdb id: 3uvx), shown in blue. The Trp residues are shown in sticks representations. BRD2(2) contains only one Trp (Trp370, structurally homologous to Trp81 of BRD4(1)); BRD4(1) presents two additional Trp residues (Trp75 and Trp120). The alignment was generated using PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

folding and ligand binding mechanisms, e.g. in the classical induced-fit model, in the case of other small protein domains involved in mediating protein-protein interactions [8] or in the case of intrinsically disordered proteins [9,10]. Interestingly, in the case of BRD domains, a ligand-induced conformational change has been proposed and discussed [11,12]. In light of these considerations we believe that obtaining information about the folding mechanism of the BRD domains may pave the way to a better understanding of their binding mechanism.

In this report we investigate the thermodynamic properties and the folding mechanism of two BET bromodomains: the second BRD of BRD2 (hereafter, BRD2(2)) and the first BRD of BRD4 (hereafter, BRD4(1)) by equilibrium spectroscopy and pre steady-state kinetic experiments. We decided to focus on these two BET BRDs because i) they are representative of the first and second domains generally found in the BET BRD family and ii) they represent ideal experimental system to investigate conservation (if any) of the folding mechanism among members of a fold family. Indeed these two BET BRDs display a 56% sequence similarity and, as can be seen from Fig. 1, they are structurally very similar (Cα root-mean square deviation (RMSD) is 1.2 ± 0.7 Å). It should be recalled that the folding mechanism of other all-α proteins has been studied in detail [13–16], leading to the hypothesis that formation of a folding intermediate is tuned by the specific α-helical propensities.

Quantitative analyses of stopped-flow (SF) mixing experiments and ultra-rapid temperature-jump (T-jump) data, allowed us to show that the folding mechanism of both BRDs are consistent with the presence of a folding intermediate, transiently populated in the sub-milliseconds time-regime. However, our results suggest that the two intermediate species show dissimilar thermodynamic and structural properties, highlighting different dynamic properties of these two BRDs.

2. Materials and methods

2.1. Protein expression and purification

BRD2(2) and BRD4(1) were expressed in *E.coli* and purified as previously described [17] and briefly reported in the legend to Fig. S1. [17]. Structural integrity of the purified proteins was checked by CD spectra in the far- and near-UV region (Figs. S2 and S3, respectively).

2.2. Urea-induced equilibrium unfolding

All experiments were carried out at 20 °C in 20 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 200 μM DTT. Intrinsic fluorescence emission measurements were carried out with a LS50B spectrofluorimeter (Perkin-Elmer) using a 1.0 cm path length quartz cuvette. Fluorescence emission spectra were recorded from 300 to 450 nm (1 nm sampling interval), with the excitation wavelength set at 295 nm. Circular dichroism (CD) measurements were performed with a JASCO J-720 spectropolarimeter using a 0.2-cm cuvette. For urea-induced equilibrium unfolding, proteins (final concentration ranging over 50.0 −100 μg/mL) were incubated at 20 °C at increasing concentrations of urea (0 − 9.5 M). When equilibrium was reached, intrinsic fluorescence emission and far-UV CD spectra were recorded in parallel. To test the reversibility of the unfolding, BRD2(2) and BRD4(1) were denatured in 7.9 M urea at protein concentration ranging over 0.5–1.0 mg/mL. After 10 min, refolding was started by 15-fold dilution of the unfolding mixture into solutions of the same buffer used for unfolding containing decreasing urea concentrations. The final protein concentration ranged over 50.0 − 100 μg/mL. After 24 h, intrinsic fluorescence emission and far-UV CD spectra were recorded at 20 °C. All equilibrium unfolding experiments were performed in triplicate. The changes in intrinsic fluorescence emission spectra at increasing urea concentrations were quantified as the changes of the relative fluorescence intensity at 345 and at 350 nm for BRD2(2) and BRD4(1), respectively. The excitation wavelength used was 295 nm.

Urea-induced equilibrium unfolding transitions monitored by far-UV CD ellipticity and intrinsic fluorescence emission changes were analysed by fitting baseline and transition region data to a two-state linear extrapolation model [18] according to

$$\Delta G_{\mathrm{unf}} = \Delta G^{H_2O} + m[\mathrm{Urea}] - RT\ln(K_{\mathrm{unf}}) \quad (1)$$

where ΔG_{unf} is the free energy change for unfolding for a given denaturant concentration, $\Delta G_2^{\mathrm{H}\circ}$ the free energy change for unfolding in the absence of denaturant and m a slope term which quantifies the change in ΔG_{unf} per unit concentration of denaturant, R the gas constant, T the temperature and K_{unf} the equilibrium constant for unfolding. The model expresses the signal as a function of denaturant concentration:

$$y_i = \frac{y_N + s_N[X]_i + (y_U + s_U[X]_i)*\exp[(-\Delta G^{H_2O} - m[X]_i)/RT]}{1 + \exp\left[\frac{-\Delta G^{H_2O} - m[X]_i}{RT}\right]} \quad (2)$$

where y_i is the observed signal, y_U and y_N are the baseline intercepts for unfolded and native protein, s_U and s_N are the baseline slopes for the unfolded and native protein, $[X]_i$ the denaturant concentration after the i^{th} addition, ΔG^{H_2O} the extrapolated free energy of unfolding in the absence of denaturant, m the slope in a $\Delta G_{\mathrm{unfolding}}$ versus [X] plot.

The denaturant concentration at the midpoint of the transition, $[\mathrm{Urea}]_{0.5}$, according to Eq. (2), is calculated as:

$$[\mathrm{Urea}]_{0.5} = \Delta G^{H_2O}/m \quad (3)$$

2.3. Kinetic experiments

Stopped-flow kinetic folding experiments were carried out on a SX-17 stopped-flow instrument (Applied Photophysics, Leatherhead, UK) in Tris/HCl 50 mM buffer pH 7.5, 0.2 M NaCl, 2 mM DTT, at 20 °C; the excitation wavelength was 280 nm and the fluorescence emission was measured using a 320 nm cut-off glass filter. In all experiments, refolding and unfolding were initiated by a 11-fold dilution of the denatured or the native protein with the appropriate buffer. Usually 4–6 individual traces were accumulated and averaged. Final protein concentration was typically 5 μM.