Slow Transition between Two β-Strand Registers Is Dictated by Protein Unfolding

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The aryl hydrocarbon receptor nuclear translocator (ARNT) is a basic helix−loop−helix Period/ARNT/Single-minded (bHLH-PAS) protein that controls various biological pathways as part of dimeric transcriptional regulator complexes with other bHLH-PAS proteins.[1–4] These complexes utilize two PAS domains, PAS-A and PAS-B, within ARNT to mediate protein/protein interactions via residues located on their β-sheet surfaces.[5,6] Site-directed mutagenesis studies of ARNT PAS-B have demonstrated the plasticity of this domain to adopt different conformations.[7] In particular, one point mutation, Y456T, on the ARNT PAS-B solvent exposed β-sheet surface resulted in two conformations ("wt" and "+3") that coexist in approximately equimolar concentrations. These conformations differ by a +3 shift in register and accompanying inversion of a central Iβ-strand along with an isomerization of the N448-P449 peptide bond in the preceding HI loop (Figure S1). Intriguingly, we found that the two conformations of Y456T interconvert slowly enough that the two forms could be separated by ion exchange chromatography.[7]
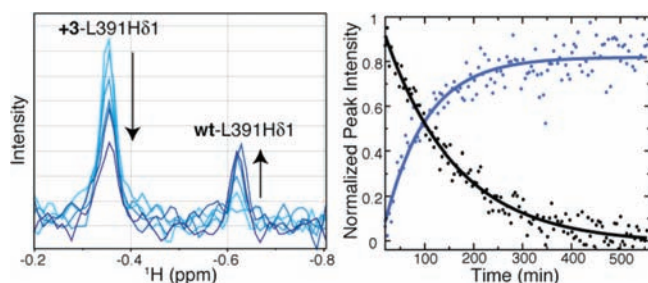

Figure 1. Monitoring the rates of interconversion for ARNT PAS-B Y456T. Left: Peak intensity changes in 1D ^{1}H NMR spectra are recorded as the protein re-establishes equilibrium ($t = 0$ min, light blue; $t = 500$ min, dark blue), as monitored by L391Hδ1. Right: Kinetic traces for the interconversion, corresponding to peak intensity changes for the wt (blue) and +3 (black) conformations.

To measure the kinetics of this interconversion process for ARNT PAS-B Y456T, we recorded a series of sequential ^{13}C-edited 1D ^{1}H experiments on a sample enriched for the +3 conformation (12:88, wt:+3) as obtained by ion exchange chromatography of a Y456T sample (51:49, wt:+3). As expected, initial spectra in these series showed peaks resulting primarily from the +3 conformation, as illustrated by the peak at −0.348 ppm, which corresponds to the Leu391-δ1 methyl group in the +3 conformation (Figure 1). This same methyl group in the wt conformation is located at −0.628 ppm and was not observed in the initial spectra. By recording a series of spectra collected sequentially, ~4 min apart, we were able to monitor the process of interconversion between the two conformations as the system relaxed back to equilibrium. We fit the time-dependent peak intensity changes from these spectra to a single exponential decay, allowing the extraction of the kinetic

rates. These analyses showed similar rates for the disappearance of +3 and concomitant formation of wt. In addition, we characterized the temperature dependence of this process between 278 and 291 K and found a linear Eyring dependence, with rates between $k = 5.49 \times 10^{-4}$ and 2.13×10^{-4} s^{-1}, respectively, Figure S2. Eyring analysis indicated that a large enthalpic barrier, 12.7 kcal/mol, must be crossed during interconversion (Table 1), most likely due to the breaking of backbone hydrogen bonds in the β-sheet. Therefore, we set out to determine the mechanism of interconversion, hypothesizing the domain may partially unfold, either locally around the Iβ-strand or more globally, to interconvert.

Table 1. Thermodynamic Parameters for ARNT PAS-B Y456T

	parameter	value
interconversion +3 → WT	$\Delta G^{\ddagger}$	18.0 kcal/mol
	$\Delta H^{\ddagger}$	12.7 kcal/mol
	$\Delta S^{\ddagger}$	−17.8 cal/mol·K (−5.3 kcal/mol @ 298 K)
unfolding	$\Delta G^{\ddagger}$	21.6 kcal/mol
	$\Delta H^{\ddagger}$	11.8 kcal/mol
	$\Delta S^{\ddagger}$	−32.9 cal/mol·K (−9.8 kcal/mol @ 298 K)

We considered two models of potential mechanisms of interconversion. The first model proposes local unfolding restricted to Iβ, while a second one alternatively involves global unfolding of the protein. In this latter case, the protein must unfold to a chiefly, unfolded state and that the sequence of the protein allows for equal probability of refolding for the Y456T point mutant, presumably with different probabilities for other variants.[7]

These models differ in their expected degree of protein unfolding, leading us to investigate the rates of global folding and unfolding using stopped-flow fluorescence in combination with Gdn·HCl. As expected, the rates of folding and unfolding at different denaturant concentrations from proteins stabilized in either conformation resulted in a chevron plot, Figure S3, with a denaturant midpoint (~2.5 M Gdn·HCl) that corresponded well to our previously determined equilibrium denaturation midpoint.[7] The wildtype (wt) and F444Q/F446A/Y456T (+3) proteins exhibited relatively slow unfolding kinetics, $k_u = 5.53 \times 10^{-4}$ and 7.47×10^{-4} s^{-1}, respectively, comparable to dozens of other similarly sized proteins that have been reported with kinetics in the $k_u = 5.85 \times 10^{-6}$ s^{-1} to 89 s^{-1} range.[8,9] Folding rates for the wt conformation ($k_f = 52.9$ s^{-1}) are slightly faster than those for the +3 conformation ($k_f = 10.6$ s^{-1}) at 298 K and extracted to denaturant-free conditions, also corresponding well to reported folding rates of comparable proteins.[9] Repeating these experiments for ARNT PAS-B Y456T, which adopts both conformations, yields similar kinetics ($k_u = 6.76 \times 10^{-4}$ s^{-1} and $k_f = 158$ s^{-1}) at 298 K with zero denaturant, indicating that Y456T folds and unfolds comparably to proteins

locked into either conformation. Eyring analysis, Figure S4, for the unfolding rates of Y456T observed between 278 and 298 K resulted in large enthalpic (11.8 kcal/mol) and entropic (-9.8 kcal/mol at 298 K) barriers to unfold (Table 1). The large entropic barrier is presumably due to a drop in water entropy upon exposure of nonpolar core residues. Interestingly, these thermodynamic values for unfolding are very similar to those measured for the interconversion of Y456T.

Comparing the rates of interconversion and unfolding, we find support for interconversion proceeding through a chiefly unfolded transition state. Notably, the rates of unfolding are approximately two times faster than the rate of interconversion, and both interconversion and unfolding processes show similar temperature dependences and equivalently comparable enthalpic changes at the transition state. We interpret the different entropic changes among the two processes as reflecting the interconversion transition state as being less unfolded than the corresponding transition state on the unfolding pathway.
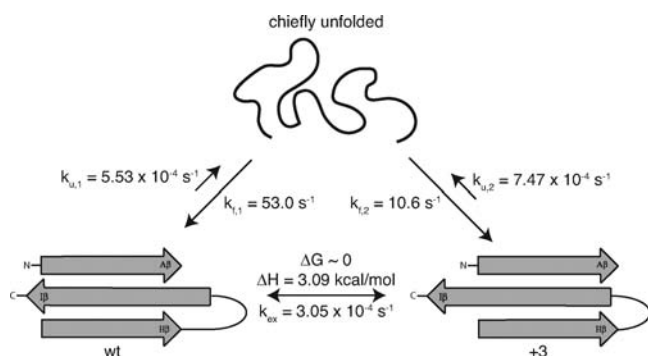

Figure 2. Summary of rates. The measured rates of interconversion are on a similar time scale as unfolding, implying the protein may undergo a global unfolding process to slip the central β-strand.

These data are consistent with the protein unfolding to a chiefly disordered state as it interconverts between conformations (Figure 2). This also supports a model of global unfolding, as the unfolded state of ARNT PAS-B Y456T has an equal probability of refolding into either folded conformation, as established by the 51:49, wt: $+3$ equilibrium. This equal probability of refolding into either conformation makes the rates of interconversion appear to be twice as slow than if the interconversion was limited to a single direction, further supporting the idea that both processes (interconversion and unfolding) transition through the same chiefly, unfolded state.

To examine a possible contributor to the slow interconversion kinetics observed in the Y456T protein, we investigated the N448-P449 peptide bond isomerization on the rates of interconversion. This peptide bond, found within the HI loop, was identified to exist in the *trans* and *cis* conformations for the wt and $+3$ proteins, respectively.[7] The *trans/cis* isomerization of X-Pro peptide bonds has been well documented as the rate-limiting step for various protein folding processes crucial for opening/closing membrane channels,[10] mediating conformer-specific ligand recognition[11] and promoting phage infection of *E. coli*,[12] all of which occur on the second to minute time scale. The enthalpy of activation ($\Delta H^{\ddagger}$) for interconversion is very close to the activation energy (E_a) of the *trans/cis* isomerization of X-Pro peptide bonds in proteins (13–20 kcal/mol).[13,14] Coupled with the difference in configuration of the N448/P449 peptide bond we observed between wt and $+3$[7], we speculated that isomerization of this bond would be involved with the rate-limiting step for the interconversion.

Previous studies of P449A/Y456T showed the protein remains well folded with the N448/P449 peptide bond in the *trans*

configuration, while the equilibrium (32:68, wt:$+3$) only slightly shifted compared to the Y456T mutant.[7] We again used ion exchange chromatography to purify a sample (>95%) in the $+3$ conformation and monitored interconversion using time-resolved NMR spectroscopy. Surprisingly, the enriched fraction did not return to equilibrium after over 300 h, indicating that this conformation is kinetically trapped, Figure S5. Notably, these samples also started to slowly precipitate 100 h postseparation. Despite this precipitation, total peak intensities in $^{15}N/^{1}H$ HSQC spectra decreased but the relative populations remained the same, indicating that the protein remained >95% in the $+3$ conformation. These data suggest once the protein folds into either conformation, it becomes kinetically trapped in that state. However, this can be escaped by denaturation in 3.5 M Gdn·HCl, letting the protein refold back to its initial equilibrium (28:72, wt:$+3$) upon its removal. To compare these interconversion results with unfolding kinetics, we used Gdn·HCl denaturation to probe the folding/unfolding rates for P449A/Y456T. Analysis of the chevron plot shows a 2-fold increase in the folding rate ($k_f = 330$ s^{-1}) and a 4-fold decrease in the unfolding rate ($k_u = 2.08 \times 10^{-4}$ s^{-1}) compared to Y456T. Taken together, these results indicated that isomerization of the N448/P449 peptide bond is critical for interconversion but has only minor effects on unfolding.

We conclude from these data that ARNT PAS-B Y456T likely interconverts by unfolding to a chiefly disordered state and refolds into both conformations with equal probability thus establishing the 51:49 (wt:$+3$) equilibrium. In addition, the P449 residue located in the HI-loop critically affects the rate of interconversion, in contrast to the modest effects it has on the equilibrium distribution of this process. While the physiological relevance of this interconversion remains to be established, this system provides a useful equilibrium model for shifts in β-strand register as observed in several biological systems.[15,16]

Supporting Information Available: Complete ref 9, protocols, and Figures S1−S6 are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA9048338