

Residual Structure Accelerates Binding of Intrinsically Disordered ACTR by Promoting Efficient Folding upon Encounter

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Abstract

Intrinsically disordered proteins (IDPs) often fold into stable structures upon specific binding. The roles of residual structure of unbound IDPs in coupling binding and folding have been under much debate. While many studies emphasize the importance of conformational flexibility for IDP recognition, it was recently demonstrated that stabilization the N-terminal helix of intrinsically disordered ACTR accelerated its binding to another IDP, NCBD of the CREB-binding protein. To understand how enhancing ACTR helicity accelerates binding, we derived a series of topology-based coarse-grained models that mimicked various ACTR mutants with increasing helical contents and reproduced their NCBD binding affinities. Molecular dynamics simulations were then performed to sample hundreds of reversible coupled binding and folding transitions. The results show that increasing ACTR helicity does not alter the baseline mechanism of synergistic folding, which continues to follow “extended conformational selection” with multiple stages of selection and induced folding. Importantly, these coarse-grained models, while only calibrated based on binding thermodynamics, recapitulate the observed kinetic acceleration with increasing ACTR helicity. However, the residual helices do not enhance the association kinetics via more efficient seeding of productive collisions. Instead, they allow the nonspecific collision complexes to evolve more efficiently into the final bound and folded state, which is the primary source of accelerated association kinetics. Meanwhile, reduced dissociation kinetics with increasing ACTR helicity can be directly attributed to smaller entropic cost of forming the bound state. Altogether, this study provides important mechanistic insights into how residual structure may modulate thermodynamics and kinetics of IDP interactions.

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Introduction

Unlike well-folded proteins, intrinsically disordered proteins (IDPs) lack stable three-dimensional structures in the unbound state under physiological conditions [1–9]. They play important roles in cellular protein–protein interaction networks and are capable of interacting with many targets with specificity [3–5,10–13]. Upon specific binding, IDPs often gain stable secondary and/or tertiary structures [11,14,15]. The molecular mechanism of how IDPs achieve efficient coupled binding and folding has been under intensive studies [15–23]. In particular, the unbound states of IDPs often contain residual structure that resembles those in the bound state [6,7,11,23–26],

although the roles of such residual structure in IDP recognition remain debatable. On one hand, residual structure may serve as initial contact points that facilitate productive binding and folding, referred to as conformational selection-like mechanisms [25]. On the other hand, conformational flexibility has been argued to be crucial for binding, and increasing the level of residual structure may reduce the association kinetics [27–29]. Instead, it has been argued that rapid folding upon encounter is critical for IDPs to achieve facile specific recognition [30,31]. The dual-transition-state theory [32] predicts that the diffusion-limited encounter rate represents an upper limit for that of IDP coupled binding and folding, which cannot be achieved unless IDPs fold rapidly upon encounter (i.e., beyond the

typical speed limit of μs^{-1} for isolated proteins [33]). If only collisions with conformers with preformed, native-like structures could lead to productive formation of the bound state (i.e., conformational selection-like mechanisms), the overall association kinetics of the coupled binding and folding process would be reduced, at least by the relative population of these pre-folded states. Curiously, it has been observed that IDPs have similar association rates compared to folded proteins [34,35]. The implication is that IDPs could fold efficiently upon encounter in general. Several features of IDPs have been argued to contribute to efficient folding upon encounter, such as small interacting domains, simple folded topologies with low contact orders, and likely an appropriate balance between residual structure and conformational flexibility [7].

In a recent NMR and stopped-flow kinetic study, lešmantavičius and coworkers [36] examined the effects of stabilizing residual helices on the association of activation domain of the activator for thyroid hormone and retinoid receptors (ACTRs) with the nuclear coactivator binding domain (NCBD) of CREB-binding protein (Fig. 1). Eight ACTR mutants were designed with varying helical propensities in the N-terminal helix (H1), without perturbing its electrostatic properties or inter-molecular interactions. Intriguingly, increased helicity of ACTR H1 was observed to accelerate the rate of association with NCBD and at the same time decelerate the dissociation rate, leading to a net stabilization of the complex. Such accelerated association induced by increasing residual helicity has also been reported for the association of KIX with c-Myb [16,37] and assembly of the spectrin tetramerization domain [38]. However, this observation is in contrast to several previous studies where the association rates are either reduced (e.g., in $p27^{\text{KIP1}}$ /cyclin A/Cdk2 [27] and pKID/KIX [28] interactions) or remain similar (e.g., PUMA binding to MCL-1 [18]) with increasing residual helicity. Mechanistically how stabilized residual helices modulate the interaction kinetics and mechanism is not clear. While the association rate constant of an ideal conformational selection process depends linearly on the pre-folded population, how residual structure could modulate an induced-folding process is not clear. As discussed in the previous work

[36], one plausible explanation is that the rate-limiting folding step occurs after an initial binding step, and the energy barrier decreases with increased helical content [36]. Another interpretation could be the existence of several parallel pathways, and increasing helical population may significantly increase the flux of conformational selection like pathways (rather than lower the barrier height) [36].

Molecular dynamics (MD) simulations could provide microscopic details necessary for unveiling the molecular mechanisms of complex coupled binding and folding processes, which often involve multiple intermediate states and parallel pathways [21–23,28,31,39,40]. However, atomistic simulations using physics-based force fields remain computationally too expensive to sample reversible binding and folding transitions to obtain reliable and statistically meaningful observations on mechanism [7]. Instead, topology-based coarse-grained (CG) modeling has been shown to offer an effective tool for mechanistic studies of coupled binding and folding of IDPs into stable complexes [28,34,39], which arguably is also governed by the minimal frustration principle of protein folding [41]. With proper calibration to balance the interplay of residual folding and intermolecular interactions, these Gō-like models have been successfully applied to several IDP complexes, and many predictions have been substantiated by independent experiments [22,28,30,42–44]. In this work, we first derive a series of Gō-like CG models that are carefully calibrated to mimic ACTR mutants with various residual helical contents and reproduce their NCBD binding affinities. Milliseconds of MD simulations were then performed to sample hundreds of reversible coupled binding and folding transitions to analyze the association kinetics and mechanism. The results show that these Gō-like models, while only calibrated based on binding thermodynamics, recapitulate the observed kinetic acceleration with increasing ACTR helicity. Mechanistic analyses reveal that pre-existing structures in ACTR do not significantly alter the baseline mechanism of its synergistic folding upon binding to NCBD. Instead, they accelerate the overall association kinetics mainly by promoting more efficient folding upon encounter.

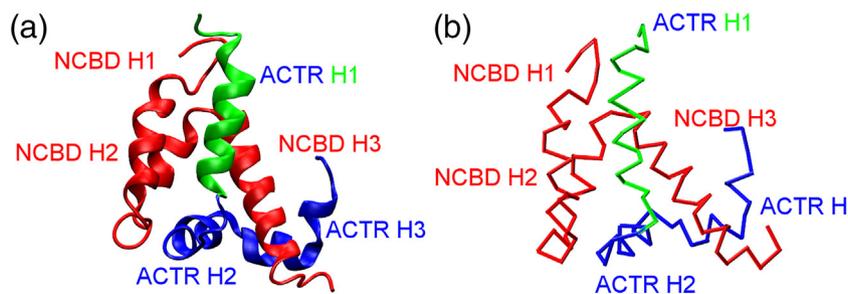


Fig. 1. (a) Structure of the NCBD/ACTR complex (PDB 1KBH [58]) and (b) the Ca-only Gō-like model.

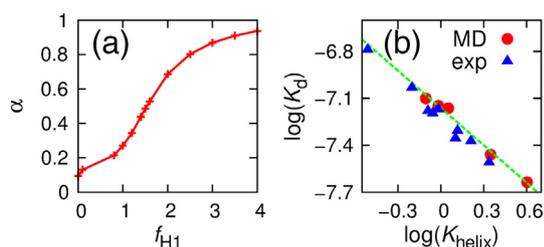


Fig. 2. (A) H1 helicity of unbound ACTR (α) at 300 K versus the scaling factor f_{H1} of intra-molecular interaction strengths within ACTR H1 region. (B) $\log(K_{helix})$ of H1 of unbound ACTR versus $\log(K_d)$ of ACTR/NCBD binding at 300 K from simulation and experiment [36]. The green line is the best-fitted line of simulation data. $K_{helix} = \alpha/(1 - \alpha)$ and simulated K_d was calculated using Eq. (1).

Results and Discussion

Gō-like models recapitulate higher NCB D/ACTR affinity with stabilized ACTR H1

As summarized in Fig. 2a and Table 1, scaling of the intra-molecular interaction strengths of ACTR H1 allows for direct modulation of its average helicity. The average helicity is bound between 10% and 95% due to the CG nature of Gō-like model. However, the resulting models overestimate how much NCB D/ACTR is stabilized by increasing ACTR H1 helicity, probably because these models under-estimate the entropic cost of binding-induced folding. To capture the binding affinities of ACTR to NCB D measured experimentally [36], the inter-molecular interaction strength between ACTR and NCB D needs to be slightly scaled down, which was determined by replica exchange (REX) simulations in combination with Hamiltonian

mapping [45,46]. As shown in Fig. 2b, $\log(K_d)$ of the final calibrated models shows a strong correlation ($R^2 = 0.98$) with $\log(K_{helix})$, as observed experimentally [36]. The slopes are also similar for the simulated and experimental values [36] (-0.80 and -0.84 , respectively). Consistently, the free energy profiles as a function of the fraction of total inter-molecular native contacts (Q_{inter}), presented in Fig. 3a and b, illustrate that the bound state becomes increasingly favorable as ACTR gains more helical structures at H1 region. Experimentally, various ACTR mutants were designed without perturbing its electrostatic interactions or inter-molecular interactions in the complex [36]. We assume that the bound state remains similar in all mutants. Therefore, increasing ACTR secondary structures could reduce the overall entropic cost of forming NCB D/ACTR complex, to result in augmented complex stability. By decomposing the free energy into enthalpic and entropic contributions (Fig. S1), we found that the overall entropic penalty ($T\Delta S$) from unbound state ($Q_{inter} = 0$) to fully bound state ($Q_{inter} = 0.6$) was indeed reduced from 78.3 to 73.4 kcal/mol as we increased ACTR H1 helicity from 0.44 to 0.80 (Fig. S1B, upper inset). Being able to capture the key features of experimentally observed structural and thermodynamic properties, the calibrated Gō-like models should enable us to investigate how residual helicity of ACTR H1 modulates the binding kinetics and pathways of NCB D/ACTR interaction.

Enhancing ACTR H1 helicity accelerates NCB D binding

An intriguing observation from the experimental study [36] is that stabilizing ACTR H1 increases the association rate constant and at the same time decreases the dissociation rate constant of ACTR/

Table 1. Summary of production simulation details and derived NCB D/ACTR binding kinetic parameters (see Methods for details)

α	f_{H1}	f_{inter}	t_{sim} (ms)	k_+ (μs^{-1})	k_- (μs^{-1})	k_{cap} (μs^{-1})	k_{esc} (μs^{-1})	k_{evo} (μs^{-1})	N_{cap}	N_{evo}	ρ_{evo} (%)	MFPT _{evo} (ns)
0.44	1.4	0.977	1.79	0.32 (0.02)	0.69 (0.04)	95.65 (0.41)	109.15 (0.55)	0.66 (0.04)	62,484	366	0.56	28.66
0.49	1.5	0.973	1.76	0.32 (0.02)	0.60 (0.03)	94.15 (0.39)	110.35 (0.43)	0.67 (0.03)	58,388	348	0.60	29.75
0.53	1.6	0.968	1.72	0.32 (0.02)	0.52 (0.03)	93.60 (0.44)	111.25 (0.49)	0.67 (0.04)	54,762	321	0.59	28.72
0.69	2	0.959	1.70	0.36 (0.02)	0.39 (0.02)	93.10 (0.52)	111.25 (0.58)	0.78 (0.03)	44,312	298	0.67	31.30
0.80	2.5	0.955	1.60	0.47 (0.04)	0.29 (0.01)	93.05 (0.70)	110.65 (0.68)	0.98 (0.08)	31,481	264	0.84	31.07

t_{sim} : accumulated simulation time from 20 independent runs.

ρ_{evo} : probability of converting collision complex to fully bound state (i.e., N_{evo}/N_{cap}).

Values in the parenthesis are standard errors of the mean, which is calculated as σ/\sqrt{n} , where σ is the standard deviation, and n is 20, that is, the number of independent runs.

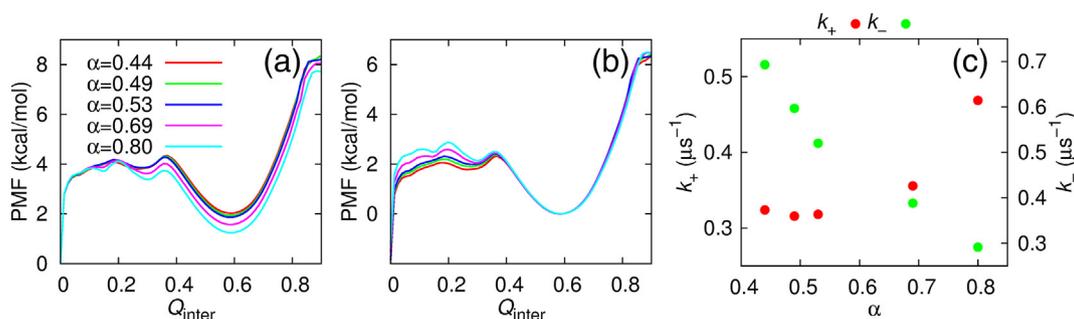


Fig. 3. (a) Potential of mean force (PMF) as a function of Q_{inter} at 314 K for five NCBD/ACTR models. These profiles were obtained from MD production simulations and calculated as $-RT \ln [P(Q_{inter})]$, where $P(Q_{inter})$ is the probability distribution of Q_{inter} , T is the temperature, and R is the gas constant. All traces have been shifted such that the free energy value at $Q_{inter} = 0$ is zero. (b) Same as panel A, except that all traces have been shifted that free energy value at $Q_{inter} = 0.58$ is zero. (c) Association and dissociation rates as a function of the H1 helicity of unbound ACTR.

NCBD complex formation [36]. Although the Gō-like models were calibrated solely based on thermodynamic properties, they can reproduce the dependence of both association and dissociation rate on ACTR H1 helicity. As shown in Table 1 and Fig. 3c, the association rate (k_+) is moderately enhanced as ACTR H1 becomes more helical, which is also consistent with decreasing binding free energy barriers as shown in Fig. 3a. Free energy decomposition analysis (Fig. S1) shows that the binding free energy barrier along Q_{inter} stemmed from the imperfect compensation of the favorable enthalpic and unfavorable entropic contributions, as predicted in the funnel-like free energy landscape theory of protein folding [47]. Stabilizing ACTR H1 results in more favorable interaction energies near the transition state (e.g., Fig. S1A inset), thus lowering the free energy barrier of association. We note that the predicted dependence of association kinetics on ACTR H1 helicity is not as linear as observed experimentally, which is likely due to the C α -only nature of current models. The simulations also predict that the dissociation rate (k_-) decreases as α is increased from 0.44 to 0.80 (Fig. 3c), which seems to be a direct consequence of increasing stability of the bound state (see Fig. 3b).

Accelerated NCBD binding through efficient folding upon encounter

To understand how ACTR H1 helicity enhancement modulates the NCBD/ACTR binding kinetics, we further evaluate its effects on various stages of NCBD/ACTR complex formation. For this, three general states, including the unbound, collision complex, and bound states, were defined [see Eq. (3)] and transitions between these states were extracted from the production trajectories and analyzed. The results are summarized in Table 1. For more disordered ACTR, there were significantly more capture events (N_{cap}), which seems to be consistent with the fly-casting theory [29]. However, the capture rate (k_{cap}) is only weakly dependent on helicity, consistent with previous analysis showing that fly-casting effects are limited for IDPs [34]. Moreover, the collision complex formation occurs considerably faster than forming the bound complex, and the correlation between capture rate and association rate is very weak with $R^2 = 0.29$ (Fig. S2), implying that this step alone unlikely determines the overall kinetics of coupled binding and folding of ACTR to NCBD. On the contrary, the evolution rate (k_{evo}) is on the same order of magnitude as the overall binding rate (k_+) (Table 1 and

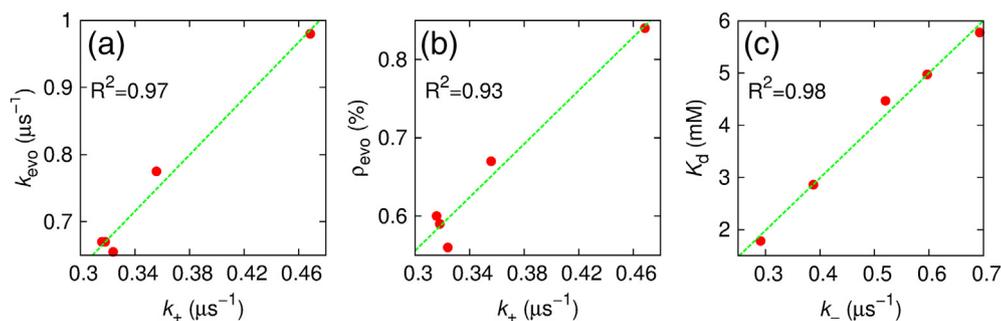


Fig. 4. Evolution rate (a) and probability of collision complex (b) evolving into the bound state as a function of the associate rate. (c) K_d at 314 K versus the dissociation rate.

Fig. 4a) and shows the strongest correlation with k_+ (Fig. 4a), suggesting that the impacts of increasing ACTR H1 helicity are mainly reflected in k_{evo} .

Note that both mean first passage time of evolution (MFPT_{evo}) and number of evolution events (N_{evo}) contribute to the calculation of k_{evo} [Eq. (6)], and we would like to further understand which one is the dominant factor that leads to increased k_{evo} (and k_+) induced by ACTR H1 stabilization. Intriguingly, MFPT_{evo} is on the order of tens of nanoseconds in all cases (Table 1), suggesting that evolution from collision complex to bound state occurs rapidly for successful transitions. This agrees well with previous notion that large-scale conformational reconfigurations of proteins are often apparently slow because such events are infrequent, and not because the actual transition processes are slow (for instance, the protein folding transition path time was found to be 10,000 shorter than the mean waiting time in unfolded state) [48–50]. This observation also implies that NCBD/ACTR complex formation seems to be a sharp, cooperative structural transition, as suggested previously by Dogan and coworkers [51]. Therefore, MFPT_{evo} cannot be used to explain the kinetic advantage of increased ACTR helicity on NCBD binding. As shown in Table 1, MFPT_{evo} indeed does not decrease when ACTR is more helical, and similar insensitivity of MFPT_{evo} in response to changes of ionic strength is also found in the case of PUMA binding to Mcl-1 [52]. Instead, greater k_{evo} and k_+ arise mainly due to the higher probability of converting collision complex to fully bound state (ρ_{evo}) for ACTR variants with more preformed H1 helical structures (Fig. 4B). That is, ACTR residual structure accelerates NCBD binding mainly by promoting more efficient folding upon encounter.

The above findings from kinetic analysis are further supported by the thermodynamic and structural analysis. As shown in Fig. 3A, the free energy profiles reveal the presence of a metastable intermediate state at $Q_{\text{inter}} \approx 0.3$, which appears to be critical for lowering binding free energy barriers as ACTR H1 helicity increases. As shown in Fig. 5, when unbound ACTR has a higher level of secondary structures, more native contacts can be formed in both peptides at this

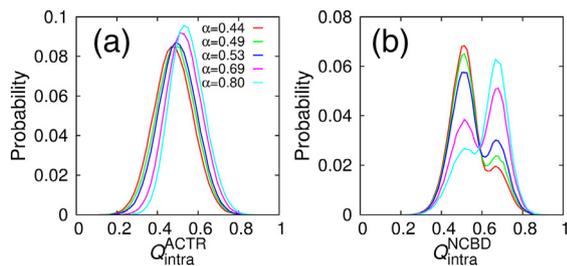


Fig. 5. Distributions of the fraction of native contacts formed within ACTR (A) and NCBD (B) in the intermediated state ($0.21 \leq Q_{\text{inter}} \leq 0.32$).

intermediate state, which may explain the extra enthalpy gain (Fig. S1A, inset) that lowers the binding free energy barrier. Furthermore, the presence of energetically more favorable, structurally more ordered intermediate state may be more “folding-competent” both energetically and topologically, which is consistent with the higher probability of collision complex evolving into fully bound state induced by ACTR helicity enhancement (Table 1). Altogether, the current analysis reinforces the pivotal role of efficient folding upon encounter in accelerating the overall kinetics of coupled binding and folding [7,30–32].

The reduced dissociation rate as ACTR H1 becomes increasingly helical (Table 1) appeared to correlate well with the increased binding affinity between ACTR and NCBD (Fig. 4C). This is not surprising considering that dissociation is largely a unimolecular process, where the rate is mainly determined by the depth of the bound free energy minimum [47]. In other words, reduction of dissociation rate is a direct consequence of stronger binding between NCBD and ACTR with increasing ACTR H1 helicity.

Baseline mechanism of NCBD/ACTR recognition

Although the topology-based models neglect many atomistic details and the absolute values presented here may not be directly compared with experimental results, these models were based on the principle of minimal frustration of protein folding [53–55] and should be able to capture the essential mechanistic features, which are referred to as the “baseline mechanism” of coupled binding and folding of IDPs. The carefully calibrated Gō-like models seem to reproduce both thermodynamic and kinetic properties of NCBD/ACTR complex formation, thus allowing us to derive further mechanistic insights into NCBD/ACTR coupled binding and folding. As shown in Fig. 6, the overall folding of each peptide is coupled with the binding in a cooperative manner, as the fractions of total intramolecular native contacts ($Q_{\text{intra}}^{\text{ACTR}}$ and $Q_{\text{intra}}^{\text{NCBD}}$) increase gradually with Q_{inter} . This is consistent with previous experimental [56] and simulation findings [22,23,31]. Such an overall mechanism is conserved as ACTR H1 becomes more helical (Fig. 6). Moreover, detailed examination of the transition path ensemble suggests that the ensemble is heterogeneous and transitions at the microscopic level may involve multiple trials and steps (Fig. 7). The presence of several intermediate states as well as involvement of both conformational selection (e.g., Fig. 7a and b) and induced fit (e.g., Fig. 7c and d) pathways seems to agree with the “extended conformational selection” model [57]. These mechanistic features have been described in detail in our previous work [22].

Stabilizing ACTR H1 results in continued differences in the pathway of coupled binding and folding. We have analyzed all productive transitions from unbound to bound states by monitoring the change

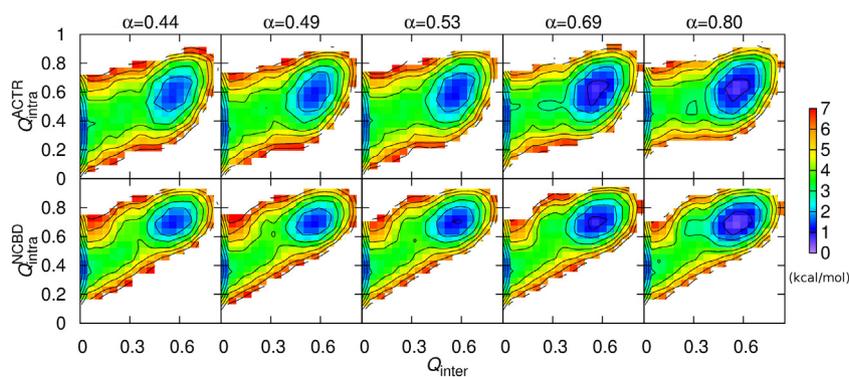


Fig. 6. 2D free energy profiles at 314 K as a function of Q_{inter} and Q_{intra}^{ACTR} (top) or Q_{intra}^{NCBD} (bottom). Contour levels are drawn at every 1 kcal/mol.

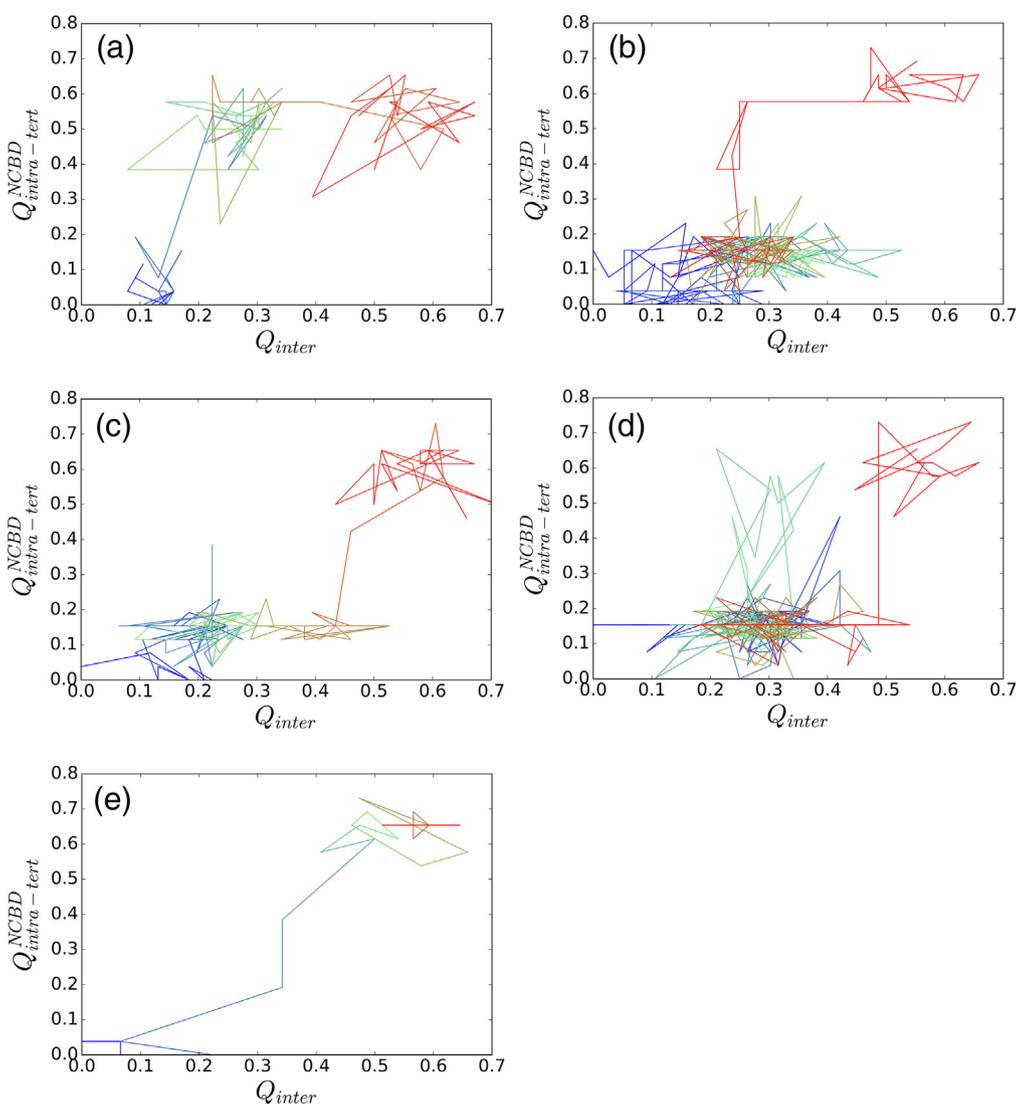


Fig. 7. Representative transitions from unbound to bound state. The traces were color in time ordering from blue to red. Panels a and b are for conformational selection-like pathways; panels c and d, for induced fit-like pathways; and e, for other pathways. Transient, but non-productive visit of alternative pathways was present in panels b and d but absent in panels a and c.

of NCBD intra-molecular tertiary structures in response to ACTR binding. Figure 8 shows the 2D free energy profiles as a function of $Q_{\text{intra-tert}}^{\text{NCBD}}$ and Q_{inter} , which reveal how the relative populations of two key intermediates (I1 and I2 in Fig. 8a) change with increasing ACTR H1 helicity. For more disordered ACTR H1, intermediate state I1 appears to be dominant, where most tertiary structures within NCBD were not formed (e.g., see Fig. 8a). As ACTR H1 becomes increasingly helical, the population of intermediate state I2 increases, where NCBD has already gained significant amount of tertiary structures when only ~25% of native contacts between NCBD and ACTR have been formed (e.g., see Fig. 8e). Such shift of relative populations of I1 and I2 also suggests that as ACTR H1 became more helical, there is a greater chance that NCBD could become folded before reach the fully bound state. This is consistent with the previous notion that enhancing the helicity of ACTR H1 results in an intermediate state with “folding-competent” topology, thus promoting efficient folding upon encounter and accelerating its association with NCBD.

Conclusions

Recent NMR structural studies and stopped-flow kinetic measurements have shown that increasing helicity of ACTR H1 not only enhances the stability of NCBD/ACTR complex but also accelerates the association rate while decelerating the dissociation rate [36]. This is surprising because previous studies have generally emphasized the importance of conformational flexibility in IDP recognition, and stabilizing residual helicities have been found to either reduce the binding kinetics or have minimal impacts [18,27,28]. Here we have constructed a series of topology-based CG models that were carefully calibrated by reproducing key thermodynamic properties of the unbound state and NCBD/ACTR interaction. Through milliseconds of MD simulations, hundreds of

reversible binding and folding transitions were generated to analyze the kinetics, thermodynamics, and mechanism of NCBD/ACTR interaction. The results show that with an increasing amount of preformed structural elements in unbound IDPs, the overall entropic cost of forming NCBD/ACTR complex is reduced, which leads to increased binding affinity and thus reduced dissociation rate. Increasing ACTR helicity does not significantly alter the baseline mechanism of the synergistic folding of ACTR and NCBD during association, which continues to follow an “extended conformational selection” model with multiple stages of selection and induced folding. Increasing residual structure in ACTR H1, however, results in a higher probability of productive evolution of nonspecific collision complexes to the final bound and folded state, and this is shown to be the primary source of accelerated association kinetics. Taken together, this study provides mechanistic insights into how residual structure modulates thermodynamics and kinetics of coupled binding and folding of IDPs and highlights the importance of efficient folding upon encounter in such processes.

Methods

Topology-based CG models of NCBD/ACTR

A C α -only sequence-flavored G \ddot{o} -like model was previously derived for the wild-type NCBD/ACTR complex (see Fig. 1) [22]. Briefly, this model was built based on PDB structure 1KBH [58] (model 1) using the MMTSB G \ddot{o} -Model Builder (<http://www.mmtsb.org>) [55,59]. It was then calibrated to recapitulate the overall residual structure level of unbound peptides and the binding affinity. This was achieved by first uniformly scaling the interaction strengths of intra-molecular native contacts, to reproduce the experimental residual helicity profiles of unbound peptides. The interaction strengths of inter-molecular native contacts were then

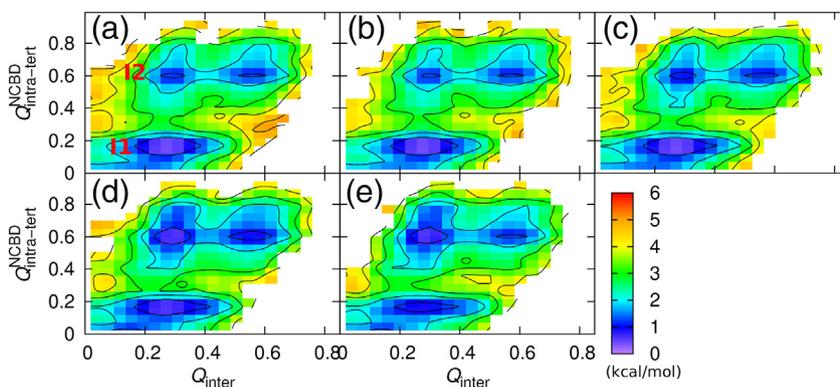


Fig. 8. Two-dimensional free energy profiles at 314 K as a function of $Q_{\text{intra-tert}}^{\text{NCBD}}$ and Q_{inter} calculated from transition path ensembles. Panels A–E are for $\alpha = 0.44, 0.49, 0.53, 0.69,$ and $0.80,$ respectively. Contour levels are drawn at every 1 kcal/mol.

scaled to match the simulated binding affinity of the complex with experimental values. In this model, the total numbers of native contacts between peptides (N_{inter}), within NCBD ($N_{\text{intra}}^{\text{NCBD}}$), within ACTR ($N_{\text{intra}}^{\text{ACTR}}$), and in NCBD intra-molecular tertiary structures ($N_{\text{intra-tert}}^{\text{NCBD}}$) are 76, 78, 49, and 26, respectively.

To model ACTR variants with different H1 residual helicities, the above Gō-like model was further tuned by uniformly scaling its interaction strengths of all intra-molecular native contacts within segment H1. Scaling factors for ACTR H1 (f_{H1}) were set at 1.4, 1.5, 1.6, 2.0, and 2.5 for five ACTR variants, which yield H1 residual helicities (α) ranging from 0.44 to 0.80 (see Table 1), comparable to the experimental values for mutants studied by Iešmantavičius and coworkers [36]. For each model, the inter-molecular interaction strengths between ACTR variant and NCBD were uniformly rescaled, such that the calculated thermodynamic stability of the NCBD/ACTR complex matched the experimental value [36]. This fine tuning was performed using REX simulations in combination with Hamiltonian mapping [45,46]. The inter-molecular scaling factors (f_{inter}) in the five final models are 0.977, 0.973, 0.968, 0.959, and 0.955, respectively (see Table 1).

Simulation protocols

All MD simulations were performed using CHARMM [60,61], and REX simulations were performed using CHARMM [60,61] with MMTSB [62]. Langevin dynamics was used with a friction coefficient of 0.1 ps⁻¹ and a time step of 10 fs. All bond lengths were constrained using SHAKE [63].

To compute the averaged helicities of ACTR H1, 1- μ s MD simulations of ACTR alone were performed at 300 K. For all simulations of NCBD/ACTR, the complex was put in a cubic box with the size of 105 Å under periodic boundary conditions. First, a REX simulation of the complex was performed for 2 μ s using the original Gō-like model [22] to generate structural ensembles. Hamiltonian mapping [45,46] was then used to identify scaling parameters that would reproduce the experimental stabilities. REX simulations (2 μ s) using these new models were then carried out to verify the stability of the complex. After model calibration, all production simulations of the NCBD/ACTR complexes were performed at 314 K, near the melting temperatures. For each model, 20 independent simulations were initiated from random conformations. The accumulated simulation times ranged from 1.60 to 1.79 ms for each complex (Table 1), which yielded hundreds of reversible binding/unbinding transitions for reliable analysis of kinetic rates and pathways. Simulation trajectories were saved every 100 ps for analysis.

Analysis

To calculate the average helicity, we first calculated the number of ($i, i + 4$) C α —C α contacts in ACTR H1. A

contact is considered to be formed if the C α —C α distance is no more than 1 Å greater than that in the fully folded state (PDB: 1KBH). The overall helicity of ACTR H1 was then calculated as the ensemble-averaged fraction of ($i, i + 4$) C α —C α native contacts formed.

For the complex, the dissociation constants (K_d in M) were computed from the bound probabilities (p_b) as [22].

$$K_d = \frac{1660 (1-p_b)^2}{V_0 p_b}, \quad (1)$$

where V_0 is the volume of simulation box in Å³. Conformations with $N_{\text{inter}} \geq 44$ are considered bound.

All kinetic information was derived from production simulations of the complex. Both the binding (k_+) and unbinding rates (k_-) were calculated directly as the inverse of corresponding mean first passage times (MFPTs) between the bound (B) and unbound (U) states [Eq. (2)], where the two states were defined as $N_{\text{inter}} \geq 44$ and $N_{\text{inter}} < 1$, respectively. Running average over 10 ns was performed before assigning the states to suppress fictitious high frequency transitions.



To further understand how residual helical stability may affect different stages of coupled binding and folding, three general conformational states were defined, including an additional collision complex (CC) state,



where k_{cap} , k_{esc} , and k_{evo} are capture, escape, and evolution rates, respectively. Here, the unbound state was more strictly defined as conformations without specific or nonspecific inter-molecular contacts (i.e., $N_{\text{inter}} < 1$ and $N_{\text{inter}}^{\text{ns}} < 1$). CC includes conformations with only nonspecific inter-molecular contacts (i.e., $N_{\text{inter}} < 1$ and $N_{\text{inter}}^{\text{ns}} \geq 1$). Again, running average over 10 ns was performed before state assignment. k_{cap} , k_{esc} , and k_{evo} were calculated from number of transitions and MFPT between states as described previously [31,34]:

$$k_{\text{cap}} = \text{MFPT}_{\text{cap}}^{-1} \quad (4)$$

$$k_{\text{esc}} = [(\text{MFPT}_{\text{esc}} \times N_{\text{esc}} + \text{MFPT}_{\text{evo}} \times N_{\text{evo}}) / (N_{\text{esc}} + N_{\text{evo}})]^{-1} \times [N_{\text{esc}} / (N_{\text{esc}} + N_{\text{evo}})] \quad (5)$$

$$k_{\text{evo}} = \left[\frac{(\text{MFPT}_{\text{esc}} \times N_{\text{esc}} + \text{MFPT}_{\text{evo}} \times N_{\text{evo}})}{(N_{\text{esc}} + N_{\text{evo}})} \right]^{-1} \times \left[\frac{N_{\text{evo}}}{(N_{\text{esc}} + N_{\text{evo}})} \right] \quad (6)$$

N_{cap} , N_{esc} , and N_{evo} are the numbers of capture, escape, and evolution transitions, respectively.

Ensembles of transition path trajectories were collected from production MD simulations to further analyze the pathways of NCBD/ACTR synergistic folding. Each transition path trajectory was defined between the fully unbound state ($N_{\text{inter}} = 0$ and $N_{\text{inter}}^{\text{ns}} = 0$) last visited and the fully bound state ($N_{\text{inter}} \geq 50$) first visited (e.g., see Fig. 7). The more stringent state criteria are necessary to eliminate spurious, noncommitting transitions. Very few intra-molecular tertiary contacts were present in bound state of ACTR [22]; thus, we did not examine ACTR folding upon binding. NCBD is a molten globular protein with a high level of secondary structures in the unbound state. Its binding-induced folding was analyzed by tracking its intra-molecular tertiary structure formation, quantified by $Q_{\text{intra-tert}}^{\text{NCBD}}$.

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Declarations of Interest: None.

Appendix A. Supplementary data

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Abbreviations used:

ACTR, activation domain of the activator for thyroid hormone and retinoid receptors; IDP, intrinsically disordered proteins; MD, molecular dynamics; MFPT, mean first passage time; NCBD, the nuclear coactivator binding domain of CREB-binding protein; REX, replica exchange.

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