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Fast association and slow transitions in the interaction between two intrinsically disordered protein domains*

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*Running title: Binding mechanism of intrinsically disordered proteins

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Keywords: intrinsically disordered proteins; binding mechanism; stopped-flow fluorescence

Capsule

Background: Intrinsically disordered proteins are common regulators of protein-protein interactions but little is known about their mechanisms of interaction.

Result: Two intrinsically disordered protein domains, from ACTR and CBP, interact through rapid association and slow conformational changes.

Conclusion: Electrostatics governs the fast association but the overall reaction is multistep.

Significance: The slow conformational search may be common among intrinsically disordered proteins with many binding partners.

SUMMARY

Proteins that contain long disordered regions are prevalent in the proteome, and frequently associated with diseases. However, the mechanisms by which such intrinsically disordered proteins (IDPs) recognize their targets are not well understood. Here, we report the first experimental investigation of the interaction kinetics of the nuclear co-activator binding domain (NCBD) of CREB binding protein (CBP) and the activation domain from the p160 transcriptional co-activator for thyroid hormone and retinoid receptors (ACTR). Both protein domains are intrinsically disordered in the free state and synergistically fold upon binding each other. Using the stopped-flow technique, we found that the binding reaction is fast, with an association rate constant of $3 \times 10^7$ M$^{-1}$s$^{-1}$ at 277 K. Mutation of a conserved buried intermolecular salt bridge showed that electrostatics govern the rapid association. Furthermore, upon mutation of the salt bridge or at high salt concentration an additional kinetic phase was detected (−20 s$^{-1}$ and −40 s$^{-1}$, respectively, at 277 K), suggesting that the salt bridge may steer formation of the productive bimolecular complex in an intra-molecular step. Finally, we directly measured slow kinetics for the IDP domains (−1 s$^{-1}$ at 277 K) related to conformational transitions upon binding. Together, the experiments demonstrate that the interaction involves several steps and accumulation of intermediate states. Our data are consistent with an induced fit mechanism, in agreement with previous simulations. We propose that the slow transitions may be a consequence of the multi-partner interactions of IDPs.

INTRODUCTION

Completely or partially disordered proteins make up a sizable fraction of proteins encoded by the eukaryotic genome (1,2). These intrinsically disordered proteins (IDPs) have rugged and flattened energy landscapes, resulting in the absence of a well-defined three dimensional structure at physiological conditions when unbound, but they often undergo a coupled folding and binding event when interacting with their ligands. IDPs have important roles in various critical cellular regulatory processes, for instance, in signaling, transcription, cell-cycle control, and translation (3,4). The abundance in the proteome, together with their functional importance, and frequent association with different types of diseases, such as cancer and neurodegenerative disorders (5), have recently sparked a
momentous interest in IDPs, which calls for a better understanding of their structural, thermodynamic, and kinetic properties (6,7).

The disordered state of IDPs has been suggested to give them certain advantages, such as the possibility of having numerous binding partners. In fact, for many IDPs, distinct structures are adopted when bound to different targets (4,8-10). It has also been theorized (11) that IDPs have a larger capture radius than ordered proteins, which would then allow for a higher association rate.

One of the most comprehensive studies on the kinetics of IDP-target interactions was conducted by Wright and colleagues a few years ago, where they used NMR relaxation dispersion experiments to investigate the coupled folding and binding of the intrinsically disordered pKID from the cAMP regulated transcription factor (CREB), to the three helix bundle KIX domain from the coactivator CBP (CREB binding protein) (12). They showed that pKID binds KIX through an induced fit mechanism, and were able to identify and characterize intermediate states along the binding reaction pathway, providing key insights into the mechanisms of molecular recognition. However, despite the growing identification of proteins that are intrinsically disordered (13), remarkably few experimental studies on the binding kinetics involving IDPs have been reported that would provide answers on the mechanisms that these proteins utilize in the interaction with their targets.

In this work we have investigated the kinetics of the specific interaction between the nuclear co-activator binding domain (NCBD) of CREB binding protein (CBP), and the activation domain from the p160 transcriptional co-activator for thyroid hormone and retinoid receptors (ACTR) (Fig. 1). Both are intrinsically disordered in the free state and synergistically fold upon complex formation to form a well-folded structure (8) with a nanomolar dissociation constant ($K_d$).

NCBD has many of the characteristics of a molten-globule, whereas ACTR is completely disordered (8,14-16). A backbone NMR relaxation study (15) showed that whereas both ACTR and NCBD exhibit substantial flexibility on the pico- to nanosecond time scale, both proteins displayed restricted backbone motions in the bound state. This results in a significant unfavorable conformational entropy change for binding, which is also reflected in the total entropy change upon complex formation, obtained from isothermal titration calorimetry (15). Clearly, disorder is important in modulating the binding free energy. However, characterization of the binding kinetics is an essential part in the elucidation of the binding mechanism of the interaction between NCBD and ACTR. Therefore, to shed light on the binding mechanism we have performed fluorescence based binding kinetic experiments. We show that the initial association between NCBD and ACTR is fast but that subsequent slow conformational changes are necessary to reach the most stable bound ground state.

**EXPERIMENTAL PROCEDURES**

**Protein expression and purification**—The DNA sequence of human ACTR (residues 1018-1088) was purchased from GENEART (Germany), while human NCBD (2058-2116) (8) was PCR amplified using a human brain cDNA library as template, and inserted into a modified pRSET vector (Invitrogen). The final construct was made up of an N-terminal hexahistidine tagged lipoyl fusion protein followed by a thrombin cleavage site (LVPRGS) and finally the ACTR or NCBD sequence. Mutants were generated by inverted PCR. *E. coli* BL21(DE3) plysS cells (Invitrogen) were grown in 2xTY medium at 37 °C and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside when OD$_{600}$ reached 0.7-0.8, to overexpress the fusion protein at 18 °C overnight. Cells were lysed by sonication, followed by centrifugation at 4 °C, after which the supernatant was passed through a 0.2 μm filter (Sarstedt), and then loaded onto a Ni-sepharose fast flow (GE Healthcare) column. After washing the column with binding buffer (40 mM Tris pH=8.0, 500 mM NaCl, 20 mM imidazole), the His-tagged fusion protein was eluted using buffer containing
250 mM imidazole. The fusion protein was then dialyzed against 20 mM Tris pH=8.0, 120 mM NaCl, after which the lipoyl protein was cleaved off using thrombin (GE Healthcare) and separated from ACTR or NCBD by loading the solution onto the Nisorparose fast flow column. The flow-through, containing ACTR or NCBD, was subjected to a reversed phase chromatography step, using C-8 (ACTR or NCBD) or C-18 (NCBD only) columns (Grace Davison Discovery Sciences). The identity of purified ACTR or NCBD was verified by MALDI-TOF mass spectrometry.

The concentration of NCBDWT and NCBDWT was determined by measuring the Trp and Tyr absorbance at 280 nm, respectively. For ACTRWT, which does not contain any Trp or Tyr, the concentration was determined by measuring the absorbance at 205 nm using an extinction coefficient obtained from amino acid analysis.

**Stopped flow measurements** - The kinetics of NCBD/ACTR association was characterized using an upgraded SX-17MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, U.K.). Measurements were performed at T=277 K or 283 K, in 20 mM sodium phosphate (pH=7.4), 150 mM NaCl. Stopped-flow experiments at high salt conditions were carried out in 20 mM sodium phosphate (pH=7.4), 0.93 M NaCl, whereas binding kinetic measurements at high TMAO concentrations were performed in 20 mM sodium phosphate (pH=7.4), 150 mM NaCl, 1 M TMAO. Excitation was at 280 nm, and the change in fluorescence upon binding was monitored using a 320 nm long-pass cutoff filter. Association rate constants (κ_{on}^{app}) were determined by varying the concentration of ACTRWT, while keeping the concentration of NCBDY2108W constant at 1 μM. In the case of ACTRQ1042W/NCBDWT and ACTRL1076W/NCBDWT, κ_{on}^{app} was determined by varying the concentration of NCBDWT, with the concentration of the Trp-ACTR variants held constant at 1 μM (277 K) or 3 μM (283 K). Overall dissociation rate constants (κ_{off}^{app}) were determined using displacement experiments. For NCBDY2108W/ACTRWT, the κ_{off}^{app} was measured by mixing a pre-formed NCBDY2108W/ACTRWT complex solution (1.1-2.2 μM NCBDY2108W mixed with 1-2 μM ACTRWT) with an excess of [NCBDWT] and monitor the change in fluorescence. For NCBDWT/ACTRWT, κ_{off}^{app} was determined by mixing a pre-formed NCBDWT/ACTRWT (2-3 μM NCBDWT mixed with 2 μM ACTRWT) with an excess of [NCBDWT]. For ACTRQ1042W/NCBDWT and ACTRL1076W/NCBDWT, the pre-formed complex solution contained 2.2 μM ACTRQ1042W or ACTRL1076W mixed with 2 μM NCBDWT, and κ_{off}^{app} was obtained by adding an excess of [ACTRWT]. The fluorescence change upon binding for the salt bridge mutants, ACTRQ1042L and NCBDG2104L, was very different from that of the ACTRWT/NCBDY2108W, and two other optical filters were used in the experiments, a 330 nm bandpass and a 355 nm cutoff filter, respectively.

**Circular dichroism spectroscopy** - CD spectra were recorded using a JASCO-810 spectropolarimeter equipped with a Peltier temperature control system. A cuvette with a path length of 1 mm was used, and far-ultraviolet spectra were recorded at T=298 K, from 260 nm to 200 nm with a scan speed of 50 nm/min, and a 2 s response time. Sample conditions were 10-23 μM protein in 20 mM sodium phosphate (pH=7.4), 150 mM NaCl. All spectra were corrected for the contribution from the buffer. CD-monitored thermal denaturation was performed by following the signal at 222 nm, using a scan speed of 1 K/min.

**Equilibrium fluorescence measurements** - Equilibrium measurements were carried out on a SLM 4800 spectrofluorimeter (SLM instruments). Experiments were performed in 20 mM sodium phosphate (pH=7.4), 150 mM NaCl. For binding experiments between NCBD and ACTR, Trp excitation was at 280 nm, and emission spectra recorded from 300 nm to 400 nm, whereas for 8-anilino-1-naphthalenesulfonic acid (ANS) (Sigma-Aldrich) fluorescence, experiments were performed at T=298 K, and excitation was at 350 nm, and the fluorescence emission recorded from 400 nm to 662.5 nm.
RESULTS
Design and validation of tryptophan variants of NCBD and ACTR-Tryptophan (Trp) residues greatly facilitate the use of fluorescence-based methods to study the kinetics of binding with high sensitivity. However, neither NCBD nor ACTR contain any Trp. Early attempts were made to see if the fluorescence of the sole tyrosine, Tyr-2108, in NCBD could be used to monitor the binding to wild-type ACTR (ACTR WT). However, only a small fluorescence change could be observed with the stopped flow technique, and a high concentration of NCBD (>10 μM) was needed to obtain reliable observed rate constants. We therefore performed a screening, where we made single amino acid substitutions, replacing a certain residue with a Trp at different locations in NCBD. These Trp variants where then evaluated on the basis of binding kinetics and of their free and bound state behavior using CD spectroscopy and equilibrium fluorescence measurements, in order to determine which of these engineered Trp variants would be most suitable as a model for wild-type NCBD (NCBD WT). Out of these, the replacement of Tyr with Trp at position 2108 (NCBD WT Y2108W) resulted in an NCBD variant that exhibited the largest fluorescence change upon binding of ACTR WT (Fig. 1, Fig. 2A). The apparent dissociation rate constant, \( k_{off, app} \), as well as its temperature dependence (data not shown), as determined by displacement experiments, was the same for NCBD WT Y2108W/ACTR WT and NCBD WT/ACTR WT (Table 1). The CD spectrum of NCBD WT Y2108W was very similar to that of NCBD WT, both in terms of shape and magnitude of the CD signal (Fig. 3A). The high similarity of the CD properties is also extended to the NCBD WT Y2108W/ACTR WT, and NCBD WT/ACTR WT complexes (Fig. 3B). Furthermore, CD-monitored thermal denaturation of NCBD WT Y2108W showed an apparent non-cooperative transition similar to that of NCBD WT (Fig. 3C), and in good agreement with previous reports (14,16). The thermal denaturations of the bimolecular complexes, NCBD WT Y2108W/ACTR WT and NCBD WT/ACTR WT, respectively, show that both have a clear transition, with a melting temperature of around 52°C, reflecting well-folded structures of the complexes (Fig. 3D).

In addition, both NCBD WT Y2108W and NCBD WT bind the fluorescent hydrophobic probe, 8-anilino-1-naphthalenesulfonic acid (ANS), resulting in an increase in fluorescence intensity and with a blue-shifted emission, which is characteristic for proteins with molten globular properties. Finally, kinetic binding experiments using high concentrations of NCBD WT and monitoring Tyr fluorescence suffered from a low signal-to-noise, but were consistent with those of NCBD WT Y2108W. Taken together, NCBD WT Y2108W proved to be a good pseudo wild type of NCBD WT and was therefore subjected to a detailed kinetic study.

We also inserted Trps in ACTR, just prior to helix 1 (ACTR WT Q1042W), or in the C-terminal helix (ACTR WT L1076W), in order to probe different regions of the ACTR/NCBD complex. The determined apparent association rate constants for ACTR WT Q1042W/NCBD WT, and ACTR WT L1076W/NCBD WT, at 277 K, were very similar to those of NCBD WT Y2108W/ACTR WT (Table 1), which further validates the use of NCBD WT Y2108W as a representation of NCBD WT.

Binding kinetics for NCBD and ACTR-We measured the kinetics of association of NCBD/ACTR using the stopped-flow technique and by varying the concentration of ACTR WT, and monitoring the fluorescence change of NCBD WT Y2108W. Alternatively, NCBD WT was varied at a constant concentration of ACTR WT Q1042W or ACTR WT L1076W, respectively. Because of the fast binding kinetics, the experiments were performed at low temperatures, 283 K and 277 K, in order to accurately determine the observed rate constant, \( k_{obs} \).

The binding kinetics of NCBD WT Y2108W/ACTR WT was biphasic with a fast phase with positive amplitude and a slow phase with negative amplitude (Fig. 4A) at 20 mM phosphate (pH=7.4), 150 mM NaCl. The concentration dependences of both kinetic phases were analyzed (Fig. 4B). The fast phase increased linearly with ACTR WT concentration with a slope of 3 × 10^7 M⁻¹ s⁻¹, which is the apparent association rate constant at 277 K (Table 1). The slow
phase remained rather constant with ACTR$_{WT}$ concentration with a rate constant $\sim$1.2 s$^{-1}$.

The apparent overall dissociation constant, $k_{off}^{app}$, was 2.6 s$^{-1}$ at 277 K (Fig. 5), as determined in a displacement experiment. In a multistep binding reaction this experimental parameter is a function of all first order rate constants, and it is equal to or smaller than the lowest microscopic off-rate constant on the reaction pathway. Biphasic dissociation kinetics were observed for the complex between ACTRL1076W and NCBD$_{WT}$, i.e., when the Trp was placed in the C-terminal helix of ACTR, with $k_{off}^{app} = 3.3$ s$^{-1}$ and 0.5 s$^{-1}$, respectively.

The initial association of NCBD and ACTR is thus very rapid, and the apparent $k_{on}^{app}$ is among the fastest that has been determined so far for an IDP system, and the first to be characterized for a system where both binding partners are IDPs. A simple extrapolation using the values of $k_{on}^{app}$ determined at 277 K and 283 K, to the physiological temperature 310 K, shows that the binding reaction approaches the diffusion-controlled limit, with a $k_{on} \sim 10^9$ M$^{-1}$s$^{-1}$. The temperature dependences of $k_{off}^{app}$ for NCBD$_{Y2108W}$/ACTR$_{WT}$, NCBD$_{WT}$/ACTR$_{L1076W}$, and NCBD$_{WT}$/ACTR$_{Q1042W}$ are all similar. The $k_{off}^{app}$ at physiological temperature (310 K) was determined to be around 130 s$^{-1}$ for NCBD$_{WT}$/ACTR$_{WT}$.

We also performed measurements at high salt concentration in order to investigate the role of electrostatics in the binding reaction. At 0.9 M NaCl, an additional kinetic phase was detected. This phase was rather constant with ACTR$_{WT}$ concentration (within the range it could be accurately fitted) with a value of $\sim$40 s$^{-1}$ (Fig. 6). Interestingly, both the fast and slow phases were not significantly affected by the addition of salt ($2 \times 10^7$ M$^{-1}$s$^{-1}$ and 0.9 s$^{-1}$, respectively). This suggested that the salt affected a particular step in the binding reaction that was too fast to detect at low salt. We note, however, that we could not detect the 40 s$^{-1}$ phase when an excess of NCBD$_{Y2108W}$ was mixed rapidly with ACTR$_{WT}$. The reason might be that the higher total fluorescence in this experiment obscured the phase.

**Binding kinetics of salt bridge mutants** - The importance of a highly conserved and buried salt bridge in the NCBD/ACTR complex, formed between D1068 in ACTR and R2104L in NCBD, has been the subject of an experimental mutational analysis by Wright and colleagues (14), where both D1068 and R2104 were mutated to leucine, thus replacing the salt bridge with a hydrophobic interaction. Further, in a recent molecular dynamics (MD) simulation study (17), it was concluded that this salt bridge stabilizes an on-pathway intermediate towards the bound state.

To test if the 40 s$^{-1}$ phase detected at high salt concentration corresponded to formation of this salt bridge we made the mutant D1068L in ACTR$_{WT}$ and R2104L in NCBD$_{Y2108W}$ and performed binding experiments at high and low salt. All three kinetic phases were affected by the D1068L/R2104L mutations. In addition, the fluorescence properties were modulated such that the sign of the amplitude for the fast phase turned negative (Fig. 7). At low salt, the concentration dependence of the first phase yielded a $k_{on}^{app}$ value of $1.5 \times 10^6$ M$^{-1}$s$^{-1}$, i.e., 20-fold lower than that of the wild type domains (Table 1). The second phase $\lambda_2$ was now clearly hyperbolic, with a $\lambda_{c}^{max}$ value of around 20 s$^{-1}$ (Fig. 7 and Table 2). The presence of the slow phase $\lambda_3$ was not clear. It showed a decrease (0.2 s$^{-1}$), along with a small amplitude, such that it approached a phase related to photobleaching. Further, fitting of all three phases simultaneously yielded a $k_{3}$ value that was much lower than $k_{off}^{app}$ from a separate displacement experiment (3 s$^{-1}$). On the other hand, fitting of a simpler two-step scheme to the salt bridge mutant gave parameters that were consistent with both $k_{off}^{app}$ and overall $K_d$ value (Table 2, Figs 7 and 2B). At 0.9 M NaCl the amplitude of the fast phase $\lambda_3$ decreased such that a quantitative analysis was difficult. However, we estimated the slope of the phase, $k_{on}^{app}$, to $0.2 \times 10^6$ M$^{-1}$s$^{-1}$, showing that other residues than D1068L in ACTR$_{WT}$ and R2104L in
NCBD<sub>Y2108W</sub> influence binding electrostatics.

It was shown previously that NCBD<sub>R2104L</sub> displays a more co-operative urea denaturation than NCBD<sub>WT</sub> (14), with an unfolding transition that occurs at about 1 M higher urea concentration. Furthermore, the magnitude of the CD signal at 222 nm of the unbound NCBD<sub>R2104L</sub> corresponded to a 25% increase in helix content compared to NCBD<sub>WT</sub>, a result that was corroborated by NMR experiments (14); thus, the ground state structures are different for NCBD<sub>WT</sub> and NCBD<sub>R2104L</sub>, which complicates the kinetic analysis. While the kinetic analysis of the D1068L/R2104L double mutation is complex, it is clear that these charged residues contribute to the high association rate constant observed for the binding of ACTR and NCBD. The most likely explanation for the appearance of the middle phase λ<sub>2</sub> is slowing down of an intramolecular step that is facilitated by formation of the salt bridge, since the phase appears both at high salt (with wild type domains) as well as upon mutation of the salt bridge.

**The order of events** - The fact that we observe more than one phase in stopped-flow experiments means that the interaction between NCBD and ACTR is not a simple one-step reaction. In a multi-step reversible reaction, such as the one under study, each constant is a complex function of all microscopic rate constants. Therefore, kinetic phases often cannot be directly assigned to a certain step. For example, the slow phase, λ<sub>3</sub>, is dependent not only on k<sub>3</sub> and k<sub>3</sub> but also on the other first order rate constants in Scheme 1 (Fig. 8). Nevertheless, the order of events may be inferred or demonstrated using different techniques.

Our data is consistent with a binding mechanism that involves at least two intermediate states, as schematically shown in Scheme 1 in Fig. 8. However, the observed rate constants are also consistent with an initial conformational change (Scheme 2 in Fig. 8, Fig. 6). If we consider the slow step as the initial one (Scheme 2), the best fit gives rate constants of approximately 1.1 s<sup>-1</sup> and 0.5 s<sup>-1</sup> for k<sub>1</sub> and k<sub>1</sub>, respectively, resulting in equilibrium concentrations of 31% of NCBD present as N and 69% as N'. The amplitude of the slow phase (negative) is 10-20% of the fast phase. Thus, if the slow phase is due to an initial conformational transition between N and N', then the fluorescence yield of N must be much larger than the fluorescence yield of N'. We rule out an initial slow step (Scheme 2) because (i) the large expected difference in fluorescence yield between the NCBD conformers is unlikely. Further, in kinetic experiments where NCBD was mixed with trimethylamine N-oxide (TMAO) we observed no slow phase, which would be expected if Scheme 2 applies; (ii) mutations in both ACTR and NCBD modulate the magnitude of the slow phase; (iii) signals in the heteronuclear single quantum coherence (HSQC) spectra are significantly broadened at lower temperatures (15,16), suggesting exchange between NCBD conformers on the µs-ms time scale, much more rapid than the observed slow phase; and (iv) the biphasic dissociation of ACTR<sub>L1076W</sub>/NCBD<sub>WT</sub> suggests that there are two conformational transitions in the complex, since ACTR is completely disordered.

The 40 s<sup>-1</sup> phase detected at 0.9 M NaCl is likely related to formation of the buried salt bridge. At low salt, this step would be rapid and not visible. With the D1068L/R2104L mutations, the 40 s<sup>-1</sup> phase is replaced by a hyperbolic phase saturating at 20 s<sup>-1</sup>, in agreement with the experiment in 0.9 M NaCl. On a kinetic basis, this phase may be related to a conformational transition occurring before or after binding. However, for the wild type domains, the presence of 1 M TMAO affects the off-rate constant significantly and only marginally k<sub>off</sub> (Table 3). If the conformational changes occurred before binding, TMAO should affect the fast equilibrium and thus the association rate constant, since TMAO selectively stabilizes more ordered structures. The lack of effect on k<sub>off</sub> also suggests that native interactions are formed late in the binding reaction (18,19). Thus, we propose that the two non-concentration dependent phases we observe are related to steps occurring after initial binding (induced fit).
DISCUSSION

IDPs may assume different structures when interacting with different ligands (20). A clear example of this structural plasticity is in fact NCBD, which adopts a three dimensional structure in its interaction with interferon regulatory factor 3 (IRF-3) (9) that has a different topology compared to the structure when bound to ACTR (8). There are also other examples where IDPs adopt distinct structures with different ligands (4,10). Further, in a recent NMR study (16), Poulsen and colleagues were able to determine the three dimensional structure of free state NCBD and showed that the conformer which was long-lived enough to be structurally characterized by NOEs, is very similar to that of NCBD when bound to ACTR, or to the transactivation domain of p53 (21), suggesting that a conformational selection mechanism is taking place. These experiments raise the question about what binding mechanisms that are involved in the recognition processes for IDPs.

Here, we directly address the reaction mechanism for the interaction between NCBD and ACTR. Our kinetic data on NCBD/ACTR demonstrate that slow conformational transitions occur after an initial rapid binding, in agreement with NMR studies on two other other IDPs, pKID/KIX (12) and p53 TAD/TAZ2 (22). Such binding mechanism is consistent with the observed structural plasticity of IDP complexes, where disordered regions search the most stable conformation with specific interactions after the initial encounter event. The presence of intermediate states is also in agreement with recent MD simulation studies (17,23) on NCBD/ACTR, where it was suggested that productive on-pathway intermediates may arise through two different pathways. One intermediate was formed by docking of the C-terminal helices, and stabilized by the highly conserved and buried salt bridge between D1068 in ACTR and R2104 in NCBD, whereas formation of the second intermediate was found to be initiated by interactions between the N-terminal helices.

While it is very difficult to experimentally distinguish binding reaction mechanisms involving several steps we can say that the simplest mechanism that is consistent with the observed kinetics is one with three consecutive steps, most probably with two conformational changes occurring after binding, as depicted in Scheme 1 (Fig. 8). The parallel pathways for initial binding suggested by the MD simulations is neither confirmed nor ruled out by our data. For example, the observed small differences in the apparent $k_{on}$ values for variants with different Trp probes (Table 1) may reflect parallel pathways for the initial encounter but could equally well be explained by mutational effects in a consecutive binding mechanism. Our data also do not rule out very rapid conformational transitions in NCBD as suggested by NMR experiments (15,16). But, if the initial binding of ACTR is to a high-energy conformer of NCBD, the microscopic $k_{on}$ must be higher than the observed $k_{on}$ (which is already very high) due to the conformational selection. Thus, it is possible that there are unbound NCBD conformers with preformed binding-competent elements that are similar to those in the bound state NCBD. The binding reaction would then involve subsequent induced fit steps, as indicated by recent molecular dynamics simulations studies (17), where the authors argued that both conformational selection and induced fit may in fact be in operation in the interaction between NCBD and ACTR, as suggested as a general mechanism (24). While we cannot rule out a fast conformational selection in the binding reaction, we can say that observable rate limiting step(s) follow the induced fit mechanism.

There are indications from previous NMR studies that different bound species that are in exchange could be present, as observed in the current study. A backbone NMR relaxation study (15) showed that, although pico- to nanosecond and micro- to millisecond ($\mu$s-$\mu$s) backbone dynamics for bound NCBD and ACTR was reduced compared to the free state, several residues for both proteins in the bound state had chemical exchange contributions to the transverse relaxation rate $R_2$, indicative of $\mu$s-$\mu$s motions and possibly the result of exchange between different states. In another study (25), the authors concluded that the NMR structure of the complex
between the activation domain of stereo receptor co-activator 1 (SRC1) and NCBD (SRC1 is a ACTR homolog), which they determined, was in exchange with another minor bound species, due to the presence of additional cross-peaks.

The removal of the buried salt bridge by mutation (NCBDR2104L and ACTRD1068L) has a profound effect on the association rate constant, reducing it by a factor of 20. IDPs tend to be enriched in charged residues, and depleted of bulky hydrophobic residues, making it difficult to form a hydrophobic core. For instance, NCBD has a total of seven Arg and Lys, and only one Asp, while ACTR has thirteen Glu and Asp and five Arg and Lys. This suggests that electrostatics may be one of the key determinants for the fast associations that have been experimentally observed for some IDPs (22). We note that, in general, $k_{on}$'s for IDPs and intrinsically disordered regions may not be larger than those of ordered proteins (18,19,26,27).

In conclusion, while our results show that initial binding could be fast for IDPs, they also highlight the disadvantage of having multiple binding partners, namely that finding the most stable conformation in the bimolecular complex may be a relatively slow process.

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**Footnotes**

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Table 1. Binding kinetics of NCBD/ACTR variants in 20 mM phosphate (pH=7.4), 150 mM NaCl at two different temperatures.

| NCBD/ACTR variant       | \( k_{\text{off app}} \) (s\(^{-1}\)) | \( k_{\text{on app}} \times 10^7 \) (M\(^{-1}\)s\(^{-1}\)) | \( \lambda_3 \) (s\(^{-1}\)) | \( k_{\text{off app}} \) (s\(^{-1}\)) | \( k_{\text{on app}} \times 10^7 \) (M\(^{-1}\)s\(^{-1}\)) | \( \lambda_3 \) (s\(^{-1}\)) |
|-------------------------|----------------------------------------|-------------------------------------------------|----------------------------|----------------------------------------|-------------------------------------------------|----------------------------|
| NCBD\( Y2108W \)/ACTR\(_{WT}\) | 2.6±0.04                               | 2.8±0.1                                         | 1.15±0.07                  | 4.5±0.04                               | 5.9±0.1                                         | 1.88±0.04                  |
| NCBD\(_{WT}\)/ACTR\(_{WT}\)       | 2.6±0.1                                | n.v.                                           | 4.4±0.5                    | n.v.                                   | n.v.                                            | n.v.                       |
| NCBD\(_{WT}\)/ACTR\(_{Q1042W}\)  | 1.5±0.1                                | 3.5±0.2                                         | n.v.                       | 2.9±0.1                                | 9.8±1.0                                         | n.v.                       |
| NCBD\(_{WT}\)/ACTR\(_{L1076W}\)  | 3.3±0.2\(^a\)                         | 2.3±0.2                                         | n.v.                       | 6.9±0.5\(^a\)                         | 7.4±0.6                                         | n.v.                       |

n.v., not visible

\(^a\)Two dissociation phases were observed.

Table 2. Estimated microscopic rate constants from global fitting of experimental binding kinetics data obtained for the NCBD\(_{R2104L}/ACTR_{D1068L}\) mutant complex at 277 K.

| Scheme (1) \(^a\) | \( k_1 \) \( \times 10^6 \) | \( k_2 \) \( \times 10^6 \) | \( k_3 \) \( \times 10^6 \) | \( k_4 \) \( \times 10^6 \) |
|-------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| Scheme (1) \(^a\) | 1.5±0.2 \( \times 10^6 \) | 48±3.7                    | 16±2.0                    | 2.8±0.2                    |
| Scheme (2) \(^a\) | 19±2.2                    | 50±3.5                    | 1.3±2.0 \( \times 10^6 \) | 2.8±0.2                    |

Units are in s\(^{-1}\), unless otherwise stated.

\(^a\) See Fig. 7B

\(^b\) Units are in M\(^{-1}\)s\(^{-1}\).

Table 3. Apparent association and dissociation rate constants of binding for NCBD\(_{Y2108W}/ACTR_{WT}\) at different solution conditions. T = 277 K.

| Solution conditions | \( k_{\text{off app}} \) (s\(^{-1}\)) | \( k_{\text{on app}} \times 10^7 \) (M\(^{-1}\)s\(^{-1}\)) |
|---------------------|----------------------------------------|-------------------------------------------------|
| 1 M TMAO \(^a\)     | 0.57±0.01                               | 4.6±0.2                                         |
| 0.9 M NaCl \(^b\)   | 1.4±0.1                                 | 1.6±0.2                                         |

\(^a\) in 20 mM sodium phosphate (pH=7.4), 150 mM NaCl

\(^b\) in 20 mM sodium phosphate (pH=7.4)

Figure Legends

Figure 1. Structure of the NCBD/ACTR complex (pdb code 1KBH). NCBD is shown in cyan and ACTR in green, with side chains of NCBD\(_{Y2108}\), ACTR\(_{L1076}\), and ACTR\(_{Q1042}\) shown in red. These residues were mutated to Trp in this study. Also shown is the buried salt bridge formed between ACTR\(_{D1068}\) and NCBD\(_{R2104}\) (blue spheres).

Figure 2. Fluorescence based equilibrium binding titration measurements at 20 mM phosphate (pH=7.4), 150 mM NaCl. Excitation was at 280 nm. A) Fluorescence emission monitored at 350 nm for NCBD\(_{Y2108W}/ACTR_{WT}\) (283 K), where NCBD\(_{Y2108W}\) was held constant at 2.8 µM at different concentrations of ACTR\(_{WT}\). B) Fluorescence emission monitored at 390 nm for NCBD\(_{R2104L}/ACTR_{D1068L}\) at 277 K, where the concentration of NCBD\(_{R2104L}\) was held constant at 2 µM, at different ACTR\(_{D1068L}\) concentrations. Data were fitted to \( F = \left( ([\text{ACTR}]_0 + K_d + [\text{NCBD}]_0) / 2 \right) \left( ([\text{ACTR}]_0 + K_d + [\text{NCBD}]_0) / 4 - [\text{ACTR}]_0 \right) \) \( \times B + C \). F is the fluorescence signal, B its total amplitude, C its intercept value, and [ACTR]_0 and [NCBD]_0 are the respective total concentrations of the ACTR and NCBD variants. Fitting was performed using Kaleidagraph (Synergy software). As seen in A) the
binding between NCBD\textsubscript{Y2108W} and ACTR\textsubscript{WT} is stoichiometric, which precludes a reliable and accurate determination of the dissociation binding constant, $K_d$. This is in good agreement with a previous report, which determined the $K_d$ to be 34 nM at 304 K, using ITC (8).

**Figure 3.** (A) CD spectra of NCBD\textsubscript{WT} (blue), NCBD\textsubscript{Y2108W} (red), and ACTR\textsubscript{WT} (green) at 298 K. (B) CD spectra of the NCBD\textsubscript{WT}/ACTR\textsubscript{WT} (blue), and NCBD\textsubscript{Y2108W}/ACTR\textsubscript{WT} (red) complexes at 298 K. (C) Thermal denaturation of NCBD\textsubscript{WT} (blue) and NCBD\textsubscript{Y2108W} (red) monitored at 222 nm. (D) Thermal denaturation of the NCBD\textsubscript{WT}/ACTR\textsubscript{WT} (blue), and NCBD\textsubscript{Y2108W}/ACTR\textsubscript{WT} (red) complexes monitored at 222 nm.

**Figure 4.** Binding kinetics of the interaction between NCBD\textsubscript{Y2108W} and ACTR\textsubscript{WT} at 20 mM phosphate (pH=7.4), 150 mM NaCl, and 277 K. (A) A typical stopped-flow trace between NCBD\textsubscript{Y2108W} (1 \mu M) and ACTR\textsubscript{WT} (6 \mu M), using a 320 nm long pass cut-off filter. Excitation was at 280 nm. The kinetics is biphasic with the inset showing the slow phase. (B) The observed rate constant for the fast (solid circles) and slow phase (solid squares) as a function of ACTR\textsubscript{WT} concentration at 277 K. The concentration of NCBD\textsubscript{Y2108W} was held constant at 1 \mu M. The fast phase $\lambda_1$ was analyzed using the general equation for association of two molecules (28). The inset shows a closer view on the concentration dependence of the slow phase, $\lambda_1$.

**Figure 5.** Dissociation kinetics at 20 mM phosphate (pH=7.4), 150 mM NaCl and 277 K. A) Example of a stopped-flow trace in a displacement experiment, where a pre-mixed NCBD\textsubscript{Y2108W}/ACTR\textsubscript{WT} (1.1 \mu M / 1 \mu M) solution was rapidly mixed with an excess of [NCBD\textsubscript{WT}] which competed out NCBD\textsubscript{Y2108W}, resulting in a single exponential fluorescence change. The residuals from the fit are shown below the trace. B) The dependence of $k_{obs}$ on NCBD\textsubscript{WT} concentration.

**Figure 6.** Binding kinetics of NCBD\textsubscript{Y2108W}/ACTR\textsubscript{WT} at 20 mM phosphate (pH=7.4), 0.9 M NaCl, and 277 K. Three phases were experimentally observed. The inset shows a closer view on the concentration dependence of the slow phase, $\lambda_3$. The dependence of the three observed rate constants on ACTR\textsubscript{WT} concentration was globally fitted to a four state sequential binding mechanism model (as illustrated in Fig. 8), in order to estimate the microscopic rate constants, using the Prism software (GraphPad). The analytical solution to the four state model is known and has been described in detail by Chemes et al. (26). A) Data fitted to a model that involves initial binding and two on-pathway intermediate states (scheme (1) in Fig. 8)). Best fit gives $k_1 = 1.6 \pm 0.2 \times 10^7$ M$^{-1}$ s$^{-1}$, $k_{-1} = 8.4 \pm 13$ s$^{-1}$, $k_2 = 40 \pm 74$ s$^{-1}$, $k_{-2} = 1.2 \pm 74$ s$^{-1}$, $k_3 = 3.9 \times 10^4 \pm 52$ s$^{-1}$, $k_{-3} = 0.8 \pm 50$ s$^{-1}$ B) Data fitted to a model involving the selection of a binding competent species followed by binding and an intermediate state (scheme (2) in Fig. 8). Best fit gives $k_1 = 41 \pm 3$ s$^{-1}$, $k_{-1} = 8.2 \pm 4.5$ s$^{-1}$, $k_2 = 1.6 \pm 0.2 \times 10^7$ M$^{-1}$ s$^{-1}$, $k_{-2} = 1.3 \pm 13$ s$^{-1}$, $k_3 = 3.2 \times 10^4 \pm 9$ s$^{-1}$, $k_{-3} = 0.8 \pm 9$ s$^{-1}$. The standard errors from the fits are large for several of the rate constants, in particular $k_3$ and $k_{-3}$, due to the lack of concentration dependence of the slower phases, $\lambda_2$ and $\lambda_3$.

**Figure 7.** Biphasic binding kinetics of the salt bridge mutant NCBD\textsubscript{R2104L}/ACTR\textsubscript{D1068L} at 20 mM phosphate (pH=7.4), 150 mM NaCl, and 277 K. A) Stopped-flow binding trace using a 355 nm long-pass cut off filter (excitation at 280 nm). 1 \mu M NCBD\textsubscript{R2104L} was mixed with 20 \mu M ACTR\textsubscript{D1068L}. Data were fitted to a double exponential function, and the residuals from the fit are shown below the trace. Global fit of the dependence of the two experimentally observed rate constants on ACTR\textsubscript{D1068L} concentration was performed in order to obtain the microscopic rate constants. In C) a model describing a two-step induced fit mechanism was employed to fit the data (panel B, scheme (1)). D) A conformational selection model (panel B, scheme (2)) was used to fit the data. The fitting was restricted by reducing the number of free variables through the use of a dissociation binding constant $K_d=5.4$ \mu M, which was determined in a separate equilibrium binding experiment (Fig. 2B). See Table 2 for the
estimated microscopic rate constants for the two models. The fitting was performed using the Prism software (GraphPad).

**Figure 8.** Schemes that were used to quantitatively describe our kinetic data. Scheme (1) involves two productive on-pathway intermediates along the binding reaction, whereas in scheme (2), there is an initial selection of a binding competent species of NCBD, followed by the formation of an on-pathway intermediate. PDB accession codes 2KKJ and 1KBH were used for the structural models of unbound NCBD\textsubscript{WT}, and the NCBD\textsubscript{WT}/ACTR\textsubscript{WT} complex, respectively. ACTR\textsubscript{WT} in the free state is completely disordered, and the schematic model of ACTR\textsubscript{WT} is shown only to visualize such a state.
Figure 1
Figure 2

A

B

Fluorescence vs. [ACTR\textsubscript{WT}] (\mu M)

Fluorescence vs. [ACTR\textsubscript{D1068L}] (\mu M)
Figure 3

(A, B) The change in optical rotation with wavelength at different temperatures. (C, D) The change in optical rotation with temperature at different wavelengths.
Figure 4

A

Fluorescence vs. Time (s)

Residual vs. Time (s)

B

Observed rate constant ($k_{\text{obs}}$) vs. [ACTR$_{\text{WT}}$] (µM)
Figure 5

A

Time (s)

Residual

Fluorescence

0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

B

$k_{obs}$ (s$^{-1}$)

$[NCBD_{WT}]$ (µM)
Figure 6

[Graph A]

- $k_{obs}$ (s$^{-1}$) vs [ACTR$_{WT}$] (µM)
- Trends for $\lambda_1$ and $\lambda_2$

[Graph B]

- $k_{obs}$ (s$^{-1}$) vs [ACTR$_{WT}$] (µM)
- Trends for $\lambda_1$ and $\lambda_2$

Inset:

- $\lambda_3$ vs [ ACTR$_{WT}$] graph
Figure 7

A

Fluorescence

Residual

Time (s)

NCBD + ACTR $\rightleftharpoons$ (NCBD:ACTR)$^*$ $\rightleftharpoons$ [NCBD:ACTR]$^{**}$ (1)

NCBD + ACTR $\rightleftharpoons$ NCBD$^*$ + ACTR $\rightleftharpoons$ [NCBD:ACTR]$^{**}$ (2)

B

C

$\lambda_1$

$\lambda_2$

D

$\lambda_1$

$\lambda_2$
Figure 8

\[ \text{NCBD} + \text{ACTR} \leftrightarrow \text{(NCBD:ACTR)} \leftrightarrow \text{(NCBD:ACTR)*} \leftrightarrow \text{[NCBD:ACTR]**} \quad (1) \]

\[ \text{NCBD} + \text{ACTR} \leftrightarrow \text{NCBD* + ACTR} \leftrightarrow \text{(NCBD:ACTR)*} \leftrightarrow \text{[NCBD:ACTR]**} \quad (2) \]
Supplemental Data

Fast association and slow transitions in the interaction between two intrinsically disordered protein domains

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Figure S1: Temperature dependence of the apparent dissociation rate constant, $k_{\text{off}}^{\text{app}}$, ranging from 277 K to 310 K. For NCBD$_{WT}$/ACTR$_{L1076W}$ biphasic dissociation kinetics was observed, of which the temperature dependence of both phases are shown here. Linear regression of the data shows that the different NCBD/ACTR variants have very similar slopes. Buffer conditions were 20 mM phosphate (pH=7.4), 150 mM NaCl.