-hub coregulator structure and flexibility determine transcription factor binding and selection in regulatory interactomes

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Published in:
Journal of Biological Chemistry

DOI:
10.1016/j.jbc.2022.101963

Publication date:
2022

Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
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Received for publication, January 25, 2022, and in revised form, April 13, 2022 Published, Papers in Press, April 20, 2022, https://doi.org/10.1016/j.jbc.2022.101963

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Edited by Ursula Jakob

Formation of transcription factor (TF)–coregulator complexes is a key step in transcriptional regulation, with coregulators having essential functions as hub nodes in molecular networks. How specificity and selectivity are maintained in these nodes remain open questions. In this work, we addressed specificity in transcriptional networks using complexes formed between TFs and αα-hubs, which are defined by a common αα-hairpin secondary structure motif, as a model. Using NMR spectroscopy and binding thermodynamics, we analyzed the structure, dynamics, stability, and ligand-binding properties of the Arabidopsis thaliana RST domains from TAF4 and known binding partner RCD1, and the TAFH domain from human TAF4, allowing comparison across species, functions, and architectural contexts. While these αα-hubs shared the αα-hairpin motif, they differed in length and orientation of accessory helices as well as in their thermodynamic profiles of ligand binding. Whereas biologically relevant RCD1–ligand pairs displayed high affinity driven by enthalpy, TAF4–ligand interactions were entropy driven and exhibited less binding-induced structuring. We in addition identified a thermal unfolding state with a structured core for all three domains, although the temperature sensitivity differed. Thermal stability studies suggested that initial unfolding of the RCD1–RST domain localized around helix 1, lending this region structural malleability, while effects in TAF4–RST were more stochastic, suggesting variability in structural adaptability upon binding. Collectively, our results support a model in which hub structure, flexibility, and binding thermodynamics contribute to αα-hub–TF binding specificity, a finding of general relevance to the understanding of coregulator–ligand interactions and interactome sizes.

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RCD1 is a member of the plant-specific SRO family and contains several domains, one of which is the RST αα-hub domain (18). Arabidopsis thaliana RCD1 (AtRCD1) plays important roles in stress responses and development (19–21), and in accordance with RCD1 functioning as a cellular hub protein (22), rcd1 knockout mutants display pleiotropic effects in stress responses and development (19). RCD1 negatively affects abiotic stress responses via RST-mediated interactions with the TF dehydration-responsive element–binding protein 2A (DREB2A) (23) and A. thaliana NAM, ATAF1/2, and CUC2 (ANAC) 013 and ANAC017 (24, 25). Biochemically, AtRCD1–RST is well characterized, and its NMR structure has been solved alone and in complex with DREB2A (14, 24), and the RCD1-binding short linear motif (SLiM) has been identified (26–28). The RST domain is also found in the plant paralogs TAF4 and TAF4b (18, 19, 29), encoded by genes with constitutive and narrow expression patterns, respectively (30). TAF4s are crucial for the structural integrity of the TFIID general TF complex (31–33). Based on the common architecture of Arabidopsis and human (Homo sapiens [Hs]) TAF4, their αα-hub domains, RST and TAFH, respectively, are likely to share the molecular function of interacting with TFs (13, 34, 35).

In this study, we addressed specificity in transcriptional networks using αα-hub–TF interactions as model invoking three different αα-hubs from three different hub proteins. Based on a comparison of their three-dimensional structures, one determined in this work, conformational stability, and binding thermodynamics, discrimination between ligands was apparent. Thus, high-affinity AtRCD1–RST–TF interactions were driven by binding enthalpy, and lower affinity TAF4–αα-hub–TF interactions were driven by entropy. This discrimination was also manifested in different degrees of folding upon binding and likely reflects specific association of biologically relevant αα-hub–TF pairs and unspecific dynamic association of “random” ligands with the TAF4 αα-hub domains. A thermal unfolding state with a substantial helical core was identified for all three domains, but with different temperature sensitivity, suggesting variability in structural adaptability relevant to binding. Together, the results revealed that αα-hub–TF interactions depend not only on coupled folding and binding of both partners but also on the formation of specific contacts, which will facilitate maximum folding, a key factor toward specificity.

Results

Domain architectures and sequences may hold clues to interactomes

For comparison of protein domains, it is important to consider them as a part of whole proteins. Figure 1A shows the domain architectures of the three αα-hub proteins AtRCD1, AtTAF4, and HsTAF4. As expected from similarities in functions, the two TAF4 proteins have similar architectures. They carry a C-terminal TAF4 domain, with the αα-hub domain located in slightly different positions within the two proteins. AtRCD1 has a different domain architecture with the RST αα-hub domain at the C terminus, and WWE (consisting of tryptophan [W] and glutamate [E] residues) polymerase domains characteristic of the SRO family, N-terminal to this (18). Functional similarities and differences are also evident from the interactomes of the three αα-hub–carrying proteins (Fig. 1B). AtRCD1 binds many different TFs (19, 26, 27), while the known interaction partners of the TAF4 proteins are mainly TFIID components, as part of the TFIID complex (32). Thus, the two TAF4 proteins have similar functions and domain architectures, whereas AtRCD1 is different, both with respect to domain architecture and interactome.

Alignment of the sequences of the three domains revealed low similarity (Fig. 1C), with AtTAF4–RST displaying 25% and 21% identity to the AtRCD1 and the HsTAF4 αα-hub domains, respectively. Previous studies identified residues involved in AtRCD1–RST interactions (14, 28). Of these, R560 and I563, which are important for DREB2A binding (14), are conserved or has conservative substitutions in both TAF4 αα-hub

Figure 1. Domain architectures, interactomes, and sequence alignments of AtTAF4, AtRCD1, and HsTAF4. A, schematic domain organization of AtRCD1 (Q8RYS9), AtTAF4 (AT5G43130), and HsTAF4 (Q00268). B, interactomes of AtRCD1, AtTAF4, and HsTAF4 obtained from the IntAct Molecular Interaction Database (60). The central αα-hub containing proteins are color coded as in A. Black interaction partners are TFs, TFIID components are orange, and other types of proteins are shown as white circles. C, sequence alignment of the AtTAF4–RST, AtRCD1–RST, and HsTAF4–TAFH αα-hub domains. Conserved residues are shown in red, and positions with conservative substitutions are shown in yellow. The secondary structure elements of AtRCD1–RST (Protein Data Bank code: 2QAO) and HsTAF4–TAFH (Protein Data Bank code: 2P6V) are shown above and below the alignment, respectively. Red dots highlight key residues for interactions between AtRCD1–RST and DREB2A TFs (14). Residue numbering is from AtTAF4. AtRCD1, Arabidopsis thaliana radical-induced cell death 1; AtTAF4, Arabidopsis thaliana transcription initiation factor TFIID-subunit 4; DREB2A, dehydration-responsive element–binding protein 2A; HsTAF4, Homo sapiens transcription initiation factor TFIID-subunit 4; TF, transcription factor.
domains. For R551, also affecting ligand binding (14), charge conservation in AtTAF4–RST (K234), but not in HsTAF4–RST (Y641), was seen. According to the structure model of the AtRCD1–RST–DREB2A complex, V547, L559, and L566 contribute to the hydrophobic ligand-binding cleft of the AtRCD1–RST αα-hub domain (14). Of these, only the position corresponding to V547 is not conserved in the two TAF4 αα-hub domains, which instead have a threonine in this position (Fig. 1C).

In conclusion, AtTAF4 has a domain architecture and interactome more similar to HsTAF4 than to AtRCD1. However, sequence comparison revealed residues conserved specifically in the RST domains, suggesting a larger degree of structure–function linkage for these domains. These similarities and differences between the three domains may determine differences in their ligand specificity.

The AtTAF4–RST structure reveals a topology similar to AtRCD1–RST and different from HsTAF4–TAFH

The three-dimensional structures of the AtRCD1 and HsTAF4 αα-hub domains are known (14, 24, 35). To obtain a structural description of AtTAF4–RST, we first recorded small-angle X-ray scattering (SAXS) data at six different concentrations (Tables S1 and S2). The shapes of the SAXS curves (Fig. S1A) and the Guinier plots (Fig. S1B) were similar, indicating the absence of aggregation. \( R_g \) (radius of gyration) and molecular weight (MW) were calculated from the Guinier plots, disregarding data from the lowest and highest concentration (Table S2). An average MW of 8.8 ± 0.4 kDa was obtained, in agreement with the expected MW of 8.9 kDa. The average \( R_g \) was 15.5 ± 0.3 Å, suggesting a slightly more compact structure than that of 16.1 ± 0.2 Å measured for the AtRCD1–RST domain (14). In addition, the pair distance distribution yielded an average maximal internal distance (\( D_{\text{max}} \)) of 44.4 ± 0.7 Å and described a typical globular protein with a short disordered tail, as evidenced by the Gaussian distribution with an asymmetric end (Fig. S1C). Finally, the Kratky plots showed a bell shape with a clear maximum indicating a globular fold (Fig. S1D).

The structure of AtTAF4–RST was solved using solution-NMR spectroscopy. A total of 1364 NMR-derived restraints, including 1248 unique distance restraints and 116 dihedral angle restraints (Table S3), were used for calculating a final set of 200 refined structures. Of these, the 20 lowest energy structures without significant violations were selected to represent the structure of the domain (Fig. 2A). The SAXS curve obtained from the 4.2 mg/l sample was fitted to a calculated scattering curve generated from the NMR ensemble using CRYsOL (part of the ATSAS package (63)) (Fig. 2B). The predicted SAXS curve fitted the experimental data well (\( \chi^2 = 1.19 \)). The comparison between the envelope and the NMR structures, with a \( \chi^2 = 1.09 \), confirmed the globular fold of the AtTAF4–RST domain with the addition of a short disordered tail, here originating from the N-terminal end (Fig. 2B).

AtTAF4–RST consists of four α-helices (H1 [F193–Q203], H2 [K206–K221], H3 [K226–V235], and H4 [D239–Q250]), as described by the secondary \(^{13}\text{C}^\alpha\) chemical shifts (Fig. 2A), typical of αα-hub domains (13, 14). The loop connecting H1–H2 consists of two residues, and one residue connects H3–H4. H2 and H3 form the characteristic αα-hairpin supersecondary structure motif consisting of two consecutive antiparallel α-helices connected by a loop (L2). As for prototypical αα-hubs, L2 is folded into the five-residue link motif \( \alpha_1^\beta_4 \) (36), with the \( \beta_3 \) position of AtTAF4–RST carrying an isoleucine (I224), as the large hydrophobic side chain interacting with side chains of the two helices (Fig. 2C). The four α-helices are organized in the characteristic L-glove (14), in which the hydrophobic surface is exposed to the solvent in an L-shape suitable for protein–protein interactions, similar to that observed in AtRCD1–RST (Fig. 2D).

Structural alignments of AtTAF4–RST (Protein Data Bank code: 7AC1) with AtRCD1–RST (Protein Data Bank code: 5OAO) (Fig. 2C; \( C^\alpha\)-RMSD = 1.1 Å [34 residues]) revealed almost identical topology and secondary structure, with 15 residues in H2, 12 in H3, and 12 in H4 for both domains, and 11 and 9 residues in H1 of AtTAF4–RST and AtRCD1–RST, respectively. The helices of HsTAF4–TAFH are longer, in particular H1, which consists of 25 residues, whereas H2, H3, and H4 consist of 17, 13, and 16 residues, respectively. In addition, HsTAF4–TAFH contains a fifth helix, H5, consisting of nine residues. The L2s are similar in lengths and positions, although the two TAF4 domains have an isoleucine in the αα-hairpin stabilizing β3 position, whereas AtRCD1–RST has a methionine. As highlighted in the sequence alignment (Fig. 1C), the positive charge of the two residues of AtRCD1–RST participating in electrostatic interactions with AtDREB2A, R560, and R551, is conserved in AtTAF4–RST as R243 (H4) and K234 (H3), respectively (Fig. 2C). Of the residues engaging in hydrophobic contacts with AtDREB2A (L513, V547, L559, I563, and L566 of AtRCD1–RST) (14), L196 (H1), L242 (H4), and L249 (H4), are conserved in AtTAF4–RST, also with respect to positions in the three-dimensional structure, whereas V547 and I563 are replaced with T230 (H3) and V246 (H4), respectively (Figs. 1C and 2C). The majority of these are located in H3 and H4. According to the AtRCD1–RST–DREB2A complex model, mainly H4 is responsible for the interaction with the ligand (14). H4 has the same orientation in AtRCD1–RST and AtTAF4–RST (Fig. 2C), suggestive of similar ligand-binding clefts. G555 was suggested to be responsible for the tight angle between H3 and H4 of AtRCD1–RST (28). This position and adjacent residues are conserved in AtTAF4–RST, whereas it has been replaced by a 6-residue loop in HsTAF4–TAFH. Likely as a result of this difference, H4 has a different orientation in HsTAF4–TAFH compared with the RST domains (Fig. 2C). Consequently, the ligand-binding cleft of HsTAF4–TAFH is different from that of the other two αα-hub domains and is located between H1 and H4 (13, 35, 37).

To further compare the structurally similar RST domains, we addressed if the dynamics of the AtTAF4–RST backbone would also align with that of AtRCD1–RST (14, 38). This was done by analyzing the longitudinal (\( R_1 \)) and transverse (\( R_2 \)) \(^{15}\text{N} \) relaxation rates and the \(^1\text{H}–^{15}\text{N} \) HetNOEs (Fig. S2). HetNOEs,
reporting on N–H bond dynamics, confirmed the folded core and dynamic flanking regions of AtTAF4–RST. R1 relaxation rates were comparable across the chain ($R_1 = 1.6 \pm 0.1 \text{ s}^{-1}$), with a similar pattern displayed by the $R_2$ relaxation rates ($R_2 = 8.7 \pm 1.1 \text{ s}^{-1}$) (Fig. S2). Elevated $R_2$ rates were observed for some residues, in particular in H1 and loop regions, indicative of chemical exchange on the millisecond timescale. Compared with the relaxation rate profiles of AtRCD1–RST (Fig. S2) (39), while mostly similar, marginally higher $R_1$ rates coupled with generally lower $R_2$ rates of AtTAF4–RST suggested a faster global tumbling rate of the AtTAF4–RST domain, possibly caused by a slightly more compact structure. This is in accordance with SAXS-derived $R_g$s and structural alignments (Fig. 2C). Elevated $R_g$s may also indicate the presence of a chemical exchange component, as previously shown in AtRCD1–RST to involve access to an unfolded excited state (38). Analysis of the relaxation rate products ($R_1R_2$) (Fig. S2D), used to decouple global tumbling effects (40), corroborated this. Both RST domains have elevated $R_2$ $^{15}$N relaxation rates of H1 residues, although only AtRCD1–RST featured the large H1 $R_2$ value for I517, in the structure positioned opposite to V554/V237, in H3/L3, which in both RST domains have large $R_2$ values (Fig. S2E). No relaxation data are available for HsTAF4–TAFH.

In conclusion, the structure of AtTAF4–RST revealed an overall topology similar to that of AtRCD1–RST with corresponding secondary structure, helix orientations, and putative ligand-binding cleft. This is in contrast to the topology of HsTAF4–TAFH, which has a fifth helix and a different H4 orientation, all suggestive of a different ligand-binding site. The two RST domains have overall similar dynamical behavior, but global differences indicate a faster global tumbling rate of the AtTAF4–RST domain, originating from a more compact overall structure.

The three aa-hub domains have a common thermal unfolding state but different thermodynamic features

Conformational stability and interactome size have been hypothesized to correlate in a way that more stable proteins
inferred smaller interactomes (41, 42). Thus, considering the differences in dynamics highlighted in the previous paragraph, we determined the conformational stability of the three domains. Their conformational stabilities were determined in chemical and thermal denaturation experiments, and the unfolding process followed by CD spectroscopy, via the change in ellipticity at 222 nm, and by two-dimensional global analysis of the change in intrinsic fluorescence (Fig. S3). While the CD experiments monitor the change in secondary structure in response to increasing temperature or urea concentration, the intrinsic fluorescence follows the chemical environment of the aromatic residues (43), typically reflecting the tertiary structures. The latter was analyzed by a two-dimensional fitting procedure that combines temperature and chemical denaturant unfolding (44). Both denaturation processes produced a sigmoidal curve characteristic of a two-state unfolding (Fig. S3). Table 1 shows the parameters determined from the experiments.

According to the two-dimensional global analyses of the unfolding reaction followed by fluorescence (Fig. 3A), the ArTAF4–RST domain had a \( T_m \) of 66 ± 2 °C and a free energy of unfolding (\( \Delta G_{DN, 298K} \)) of 5.9 ± 1.3 kJ mol\(^{-1}\), suggestive of a low stability. The parameters determined by CD spectroscopy were \( T_m = 68 ± 3 °C \) and \( \Delta G_{DN, 298K} = 7 ± 2 \) kJ mol\(^{-1}\). The \( m \) values, proportional to the change in solvent-accessible surface area upon unfolding (45), were 3.7 ± 0.7 kJ mol\(^{-1}\) M\(^{-1}\) and 3.4 ± 0.3 kJ mol\(^{-1}\) M\(^{-1}\), determined by CD and fluorescence spectroscopy, respectively. These values correspond to the unfolding of approximately 45 residues and exposure of 3300 Å\(^2\) surface (45). A small unfolding heat capacity change (\( \Delta C_p \)) of 3.1 ± 0.6 kJ mol\(^{-1}\) K\(^{-1}\) is in accordance with the high \( T_m \) despite the low unfolding energy, since it causes a decrease in the temperature dependence of the stability (Fig. 3A).

For comparison, we determined the parameters for unfolding of the HsTAF4–TAFH and ArTCD1–RST domains (Table 1 and Fig. S3). Figure 3A shows the three stability curves obtained from the two-dimensional global analyses. The global stability, \( \Delta G_{DN} \), of the two αα-hub domains from the TAF4 proteins, had similar temperature dependence, described by the unfolding \( \Delta C_p \)s, but with HsTAF4–TAFH having a higher enthalpy change, \( \Delta H_m \), in accordance with the increased stability of HsTAF4–TAFH compared with that of ArTAF4–RST. ArTCD1–RST had a \( \Delta H_m \) similar to that of ArTAF4–RST, but a lower \( \Delta C_p \) of 1.8 ± 0.3 kJ mol\(^{-1}\) K\(^{-1}\), reflecting the lower temperature dependence of \( \Delta G_{DN} \). The \( \Delta H_m \) was larger for HsTAF4–TAFH than for both RST domains because of more folded residues in this larger domain. This difference also resulted in a higher \( T_m \) of 71 ± 3 °C for HsTAF4–TAFH compared with 66 ± 2 °C for ArTAF4–RST and 59 ± 5 °C for ArTCD1–RST (Fig. 3A).

From the CD experiments, HsTAF4–TAFH also had a higher \( T_m \) = 74.1 ± 0.4 °C, compared with \( T_m = 68.8 ± 0.2 °C \) for ArTCD1–RST and \( T_m = 68 ± 3 °C \) for ArTAF4–RST, respectively. A similar order was observed when comparing \( \Delta G_{DN, 298K} \) values; those of the RST domains were similar, whereas the TAFH domain had significantly larger \( \Delta G_{DN, 298K} \). The smallest \( m \) values were obtained for ArTCD1–RST and ArTAF4–RST, being 2.7 ± 0.3 kJ mol\(^{-1}\) M\(^{-1}\) and 3.8 ± 0.7 kJ mol\(^{-1}\) M\(^{-1}\), respectively, with a higher \( m \) value of 5.5 ± 0.2 kJ mol\(^{-1}\) M\(^{-1}\) for HsTAF4–TAFH, indicating a larger change in solvent-accessible surface area upon unfolding (Table 1). Although the two types of experiments do not measure the same features, there is accordance between the results obtained (Table S4).

The analyses showed that the \( \Delta Hs \) extracted from the two different unfolding experiments were different. For all three domains, the van’t Hoff enthalpy change, \( \Delta H_{vH, s} \), determined from the CD thermal denaturation was lower than the enthalpy change determined using the two-dimensional global analyses, \( \Delta H_m \) (ratios ~0.5, Tables 1 and S4), suggesting incomplete thermal unfolding and thus the presence of a putative alternative state for all three αα-hub domains. To explore this further, and to obtain structural information on this alternative state, we obtained CD spectra before and after chemical and thermal denaturation and observed the existence of pronounced residual helical structure at high temperature. In contrast, the domains were completely denatured in 8 M urea (Fig. 3B). We calculated the percentage helicity of the three domains at 20 °C and compared with those calculated for the denatured states. At 20 °C, the domains were ~50%

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| Stability of αα-hub domains |

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<tr>
<th>Domain/method</th>
<th>( T_m ) (°C) (\alpha)</th>
<th>( \Delta H ) (kJ mol(^{-1})) (\alpha)</th>
<th>( \Delta C_p ) (kJ mol(^{-1}) K(^{-1})) (\alpha)</th>
<th>( m ) (kJ mol(^{-1}) M(^{-1})) (\alpha)</th>
<th>( \Delta G_{DN} ) (kJ mol(^{-1})) (\alpha)</th>
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<tr>
<td>ArTAF4–RST</td>
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<tr>
<td>CD chemical denaturation</td>
<td>68 ± 3</td>
<td>46 ± 6</td>
<td>3.7 ± 0.7</td>
<td>7 ± 2</td>
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<tr>
<td>CD thermal denaturation</td>
<td>66 ± 2</td>
<td>117 ± 26</td>
<td>3.1 ± 0.6</td>
<td>3.4 ± 0.3</td>
<td>5.9 ± 1.3</td>
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<tr>
<td>Two-dimensional global analysis</td>
<td>68.8 ± 0.2</td>
<td>54.8 ± 0.9</td>
<td>2.7 ± 0.3</td>
<td>7.0 ± 0.6</td>
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<tr>
<td>ArTCD1–RST</td>
<td></td>
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<tr>
<td>CD chemical denaturation</td>
<td>59 ± 5</td>
<td>118 ± 13</td>
<td>1.8 ± 0.3</td>
<td>3.8 ± 0.2</td>
<td>8.8 ± 1.4</td>
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<tr>
<td>CD thermal denaturation</td>
<td>74.1 ± 0.4</td>
<td>55 ± 2</td>
<td>5.5 ± 0.2</td>
<td>13.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Two-dimensional global analysis</td>
<td>71 ± 3</td>
<td>167 ± 16</td>
<td>3.4 ± 0.1</td>
<td>4.1 ± 0.1</td>
<td>12.9 ± 1.4</td>
</tr>
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Chemical denaturation was performed by increasing the concentration of denaturant from 0 to 8 M urea, whereas thermal denaturation was performed by increasing the temperature from 15 to 90 °C. The thermodynamic parameters were calculated using Equations 2 (CD) and 3 (two-dimensional global analysis). The values are averages and standard deviations of three independent experiments.

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\( \alpha \) In the absence of denaturant.

\( b \) \( \Delta H \) corresponds to the \( \Delta H_{vH, s} \) for the CD thermal denaturation and \( \Delta H_m \) for two-dimensional global analysis.

\( c \) Value calculated at 25 °C.
helical (43% for AtRCD1–RST, 49% for AtTAF4–RST, and 52% for HsTAF4–TAHF). In the presence of 8 M urea, all domains were extensively unfolded with only ~3% helicity. However, at 80 °C in the thermally denatured states, the domains retained ellipticity at 222 nm corresponding to 17%, 23%, and 25% helical structure for AtRCD1–RST, AtTAF4–RST, and HsTAF4–TAHF, respectively. Previous observations indicate that thermally and chemically denatured protein may differ because of subensembles populated at high temperatures (46, 47). However, for the TAF4 α-hub domains, a comparably more negative ellipticity than observed for thermal denaturation in general (47) indicates retention of some α-helical structure in the unfolded state.

To further address the characteristics of the alternative state, and since hydrogen-exchange kinetics of the AtRCD1–RST domain has been shown to be extremely fast (38), we recorded series of 15N-heteronuclear single quantum coherence (HSQC) spectra of AtTAF4–RST and AtRCD1–RST over a temperature range from 25 to 55 °C (Fig. S4). For AtTAF4–RST, peak intensities increased considerably up until 40 to 45 °C (Fig. 3C). Although surprising, this matched the relative temperature independence of ΔG_DN, in this temperature range (Fig. 3A), resulting in limited unfolding but faster global tumbling, which produced sharper peaks in the HSQC spectra. At 45 to 55 °C, most peaks lost intensity although the majority of AtTAF4–RST peaks could be assigned even at the highest temperature. The behavior of AtRCD1–RST was distinctly different with most residues losing peak intensity as temperature increased above 30 °C, and no peaks were visible at temperatures above 50 °C (Fig. 3C). Interestingly, peaks belonging to residues in H2 and H3, constituting the α-hairpin, were more temperature resistant than peaks from the flanking helices. This suggested that the hydrogen bonds of the H2 and H3 were retained at higher temperatures, thus limiting solvent proton exchange of the backbone amide groups. A similar pattern was not immediately apparent for AtTAF4–RST. However, mapping of the highest temperature for which a peak from a particular residue was visible (Fig. 3D) revealed that peaks from solvent-exposed residues, particularly of H1 and H4, generally
disappeared at lower temperature, whereas peaks from residues facing the “interior” of the protein were visible at higher temperatures. This effect was also seen for AtRCD1–RST, although to a lesser extent.

Based on the results presented previously, three conclusions can be made. First, HsTAF4–TAFH is the most stable domain, most likely because of a larger buried surface area and a higher number of folded residues. Second, the unfolding $\Delta C_{p}$ of AtRCD1–RST is smaller than that of AtTAF4–RST even though they are structurally and sizewise similar. Finally, the discrepancies between $\Delta H_m$ and $\Delta H_{\text{vH}}$ values, together with the presence of residual structure after thermal unfolding, suggested the existence of a general $\alpha$-hub thermal unfolding state. This supports the presence of stable cores, inferred from the NMR data to be comprised primarily of H2 and to some extent H3, organizing more dynamic flanking helices.

**AtTAF4–RST interacts with the AtRCD1 ligands AtDREB2A and ANAC013**

AtDREB2A has previously been identified as an AtRCD1–RST interaction partner (19, 26, 27, 48). Based on the similarities of the structures and conservation of key ligand-interacting residues, we hypothesized that AtTAF4–RST would also bind AtDREB2A. Using isothermal titration calorimetry (ITC), the interaction between an AtDREB2A peptide DREB2A$_{243-272}$ containing the RCD1-binding SLiM (27) and AtTAF4–RST, was analyzed. We performed two sets of experiments; one at 25 °C (Fig. S5), which suggested an interaction between AtTAF4–RST and DREB2A$_{243-272}$ but with a small change in binding enthalpy, and another at 30 °C, which confirmed binding and had an increased contribution from $\Delta H$ and thus an improved signal-to-noise ratio (Fig. 4A). The $K_d$ for the complex of DREB2A$_{243-272}$ with AtTAF4–RST were at 25 and 30 °C 740 ± 300 nM and 1050 ± 140 nM, respectively. The interactions were characterized by a low enthalpic contribution ($\Delta H = -5.19 \pm 0.03$ kJ mol$^{-1}$ at 25 °C and $-10.5 \pm 0.2$ kJ mol$^{-1}$ at 30 °C) and were in both cases dominantly driven by entropy ($\Delta T \Delta S = -29.8$ kJ mol$^{-1}$ at 25 °C and $-24.2$ kJ mol$^{-1}$ at 30 °C) (Table 2 and Fig. 4B). The significant difference in entropy between the AtTAF4–RST and AtRCD1–RST interactions with AtDREB2A suggested that the AtTAF4 interaction involved less structuring than the AtRCD1 interaction.

To characterize the interaction between AtTAF4–RST and DREB2A$_{243-272}$ at the residue level, we used NMR spectroscopy (Fig. 4C). AtTAF4–RST was in fast-intermediate exchange between free and bound states on the NMR timescale enabling assignment of the bound state. Most AtTAF4–RST residues were affected by binding, suggesting binding to be accompanied by small structural rearrangements or stabilization of the AtTAF4–RST $\alpha$-helices, as seen previously for...
AtRCD1–RST (14). For AtRCD1–RST, most key residues for binding of AtDREB2A map to H3 and H4 (13, 14). Comparison of free and bound states of AtTAF4–RST revealed larger chemical shift perturbations (CSPs) in H3 and H4, suggesting that for AtTAF4–RST, these regions are also involved in binding, further supporting the ligand-binding cleft shown in Figure 2D. However, the CSPs were generally smaller for the AtTAF4–RST interaction than for the interaction of AtRCD1–RST, suggesting that AtTAF4–RST undergoes reduced structural changes upon AtDREB2A binding.

NMR spectroscopy was used to study the structural features of the AtTAF4–RST–bound state of DREB2A243–272 (Fig. S6). Residues from D257 to R266 showed large CSPs upon binding (Fig. 4D), similar to the results described for the AtRCD1–RST–DREB2A complex (27). Secondary 13C chemical shifts indicated helical structure in the bound-state DREB2A2 peptide (Fig. 4E). However, the AtTAF4–RST binding-induced α-helix was shorter than when in complex with AtRCD1–RST (14, 39). In addition, the secondary chemical shift of the highly conserved F259 (28), which forms extended structure in complex with AtRCD1–RST (39), did not exhibit the same behavior in complex with AtTAF4–RST. Peaks belonging to residues M258 and F259 were very weak or nonexistent indicating that the two residues were dynamic in the AtTAF4–RST complex. This is similar to what was observed for a shorter, lower affinity, DREB2A255–272 fragment in complex with AtRCD1–RST (14, 39). Together, this suggested that for DREB2A243–272, binding to AtTAF4–RST induced helical structure in the 261 to 264 region of the peptide, while the residues surrounding this central helical turn remained unstructured, a clear contrast to the interaction with AtRCD1–RST.

Both thermodynamics parameters and the secondary chemical shifts indicated less structuring of DREB2A243–272 in the AtTAF4–RST interaction compared with the interaction with AtRCD1–RST. To rationalize the different potential for structuring of the two RST domains, we analyzed the NMR unfolding CSPs in context of their respective thermal stabilities (Figs. 3A, S4 and Table 1). Based on ΔGDN temperature dependence (Equation 6, no denaturant), we determined temperature ranges resulting in similar degrees of unfolding (30–50 °C for AtTAF4–RST and 35–45 °C for AtRCD1–RST). If the domains contain structure with lower stability, we expect adjacent residues to experience increased CSPs in the selected temperature range. The extracted CSPs were generally larger for AtRCD1 than for AtTAF4. In addition, AtRCD1–RST showed a clear increase in CSPs for residues located in the interface between H1 and the remaining folded domain (Fig. 5B). For AtTAF4–RST, the CSPs were less localized. Comparison of the initial unfolding of the two domains suggested that H1 of AtRCD1–RST was more sensitive to temperature and thus also more malleable. This malleability would allow AtDREB2A to induce specific complementary structure, thus increasing the favorable enthalpic contribution at an entropic cost.

We then examined how the differences in topology and the lack of AtRCD1–RST key residues (Figs. 1C and 2C) in HsTAF4–TAFH would affect binding to DREB2A243–272 (Figs. 4B and S5B; Table 2). Surprisingly, the affinity of HsTAF4–TAFH for DREB2A243–272 was higher (Kd = 110 ± 50 nM at 25 °C) than that of AtTAF4–RST (Kd = 740 ± 320 nM) and was driven by both enthalpy and entropy, with the largest contribution stemming from entropy changes. The binding cleft of the TAFH domain differs from that of the two RST domains by being located between H1 and H4, rather than between H3 and H4 (37). Formation of a coactivator–TF complex may thus in this case be explained by stochastic burial of hydrophobic residues and unspecific electrostatic interactions, as commonly assumed for such interaction pairs (49).

Finally, we measured the binding of AtTAF4–RST to the AtANAC013 peptide, ANAC013254–274. This peptide also contains the AtRCD1-binding SLiM but behaves structurally differently from DREB2A243–272, with no detectable α-helix induction upon binding to AtRCD1–RST (27). In this experiment, the ΔH measured at 25 °C by ITC was too low for detection (Fig. S5C), but the experiment performed at 30 °C (Fig. 4, A and B) allowed determination of the thermodynamic parameters associated with binding. AtTAF4–RST bound ANAC013254–274 with Kd = 1080 ± 630 nM, a small enthalpic contribution (ΔH = −1.8 ± 0.1 kJ mol⁻¹), and a large favorable contribution from entropy change to binding (−ΔTAS = −32.7 kJ mol⁻¹) (Table 2).

To conclude, the interactions between AtTAF4–RST and two AtRCD1-binding TFs were mainly driven by favorable changes in entropy, which is in contrast to their AtRCD1–RST

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**Table 2**

<table>
<thead>
<tr>
<th>Syringe/cell</th>
<th>Temperature (°C)</th>
<th>Kd (nM)</th>
<th>N</th>
<th>ΔH (kJ mol⁻¹)</th>
<th>−ΔTAS (kJ mol⁻¹)</th>
<th>ΔG (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtTAF4–RST/DREB2A</td>
<td>25</td>
<td>740 ± 320</td>
<td>1.24 ± 0.04</td>
<td>−5.19 ± 0.03</td>
<td>−29.8</td>
<td>−35.0</td>
</tr>
<tr>
<td>AtTAF4–RST/DREB2A</td>
<td>30</td>
<td>1050 ± 140</td>
<td>1.18 ± 0.01</td>
<td>−10.5 ± 0.2</td>
<td>−24.2</td>
<td>−34.7</td>
</tr>
<tr>
<td>AtRCD1–RST/DREB2A</td>
<td>25</td>
<td>16 ± 1</td>
<td>0.90 ± 0.00</td>
<td>−63.5 ± 0.2</td>
<td>18.7</td>
<td>−44.6</td>
</tr>
<tr>
<td>HsTAF4–TAFH/DREB2A</td>
<td>25</td>
<td>110 ± 50</td>
<td>1.15 ± 0.02</td>
<td>−15.4 ± 0.4</td>
<td>−24.1</td>
<td>−39.5</td>
</tr>
<tr>
<td>HsTAF4–TAFH/DREB2A</td>
<td>30</td>
<td>420 ± 120</td>
<td>0.85 ± 0.02</td>
<td>−23.9 ± 0.7</td>
<td>−12.9</td>
<td>−36.9</td>
</tr>
<tr>
<td>AtTAF4–RST/ANAC013</td>
<td>25</td>
<td>NB</td>
<td>NB</td>
<td>−1.8 ± 0.1</td>
<td>−32.7</td>
<td>−34.5</td>
</tr>
<tr>
<td>AtTAF4–RST/ANAC013</td>
<td>30</td>
<td>1080 ± 630</td>
<td>0.68 ± 0.04</td>
<td>−45.0 ± 0.8</td>
<td>−0.6</td>
<td>−45.6</td>
</tr>
<tr>
<td>AtRCD1–RST/ANAC013</td>
<td>25</td>
<td>9 ± 4</td>
<td>0.80 ± 0.01</td>
<td>−45.0 ± 0.8</td>
<td>−0.6</td>
<td>−45.6</td>
</tr>
</tbody>
</table>

Abbreviation: NB, no detectable binding.

Syringe/cell indicates whether the α-hub domain or the TF is the titrant in the syringe or the titrant in the cell. The standard errors for ΔH, Kd, and N were obtained from Origin when fitting the data to a model of one set of binding sites.

* Data from Ref. (28).

[1] Data from Ref. (27).

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The model of α-hubs: Correlating structure, stability, and interactome.
interactions. For the HsTAF4–TAFH–DREB2A243–272 interaction, entropy also gave the largest contribution to binding at 25 °C (Table 2). Structural analysis suggested that although DREB2A243–272 undergoes coupled folding and binding in its interaction with AtTAF4–RST, the resulting α-helix is shorter than in the AtRCD1–RST complex.

**Discussion**

In this work, we have asked which properties within inter- 
actomes are important for selectivity and specificity. To address this, we have investigated the AtTAF4–RST αα-hub domain and compared it with two other αα-hub domains, one from the same species and one from humans. AtRCD1–RST was included because it is also an RST domain (13, 48), but its parent protein, AtRCD1, belongs to a different functional family than TAF4 (18). HsTAF4–TAFH was therefore also included, as it represents a TAF4 protein, but from a different species (Fig. 1A). Evolutionarily, the RST and TAFH domains differ from the paired amphipathic helix, harmonin–homology domain, and nuclear coactivator–binding domain αα-hubs by having an intron just before the region encoding the α1–β4 motif (13). Despite this, there is no evidence of a common ancestor, and thus the evolutionary relation between the two genes remains uncertain.

As a first step, the three-dimensional structure of AtTAF4–RST was determined and compared with the structures of the other two αα-hubs. The two RST domains were structurally similar forming an L-glove fold with four helices of similar lengths and the linker between H2 and H3 forming the α1–β4 motif (Fig. 2C). The RST domain structures are different from that of HsTAF4–TAFH with respect to the orientation of H4. Together with the different lengths of H1, the varying H4 orientations represent the distinctive features of different αα-hub subgroups (13, 14). Noteworthy, this changes the binding surface of HsTAF4–TAFH compared with the two RST domains (Fig. 2, C and D). The AtRCD1–RST–DREB2A complex is stabilized by residues L513, R543, V547, R551, L559, R560, I563, and L566 of AtRCD1–RST (14). All these positions, except V547, are conserved or have conservative substitutions in AtTAF4–RST (Figs. 1C and 2C). Thus, the lack of specific charged and hydrophobic residues does not explain the difference in affinities between the two RST do- 
mains, suggesting that specificity is acquired from differences in other properties of the two domains.

The conformational stability of protein hubs has recently been hypothesized to be important for their functions as exemplified by a correlation between malleability and promiscuity (49, 50). Analyzing the stability and folding thermodynamics of the three αα-hubs, we found that the domains populate a common alternative state at high temperatures with relatively high content of helicity (Fig. 3C and Table 1). It is possible that this state represents the unfolded excited state, recently identified for AtRCD1–RST (38), but more elaborate studies are needed to fully confirm this. Previous studies on the dynamics and stability of αα-hubs suggested that the H2–H3 αα-hairpin comprises a relatively rigid structural element, whereas the other helices, in particular the C-ter- 
iminal region of H1, are more flexible (13). Thermal denaturation using CD and NMR spectroscopy corroborated this (Fig. 3, B–D). Although similarities concerning the unfolding state can be identified, our data indicate that the two RST domains behave differently when exposed to higher temperatures, with AtTAF4–RST being relatively rigid and maintaining its structure, whereas AtRCD1–RST has more flexible flanking helices that unfold at lower temperatures. We suggest that these features provide the foundation of the larger interac- 
tome of AtRCD1–RST as it allows the hub protein to adapt and bind an increased number of interaction partners with higher af- 
finity (Fig. 6).

Heat capacity depends on many parameters including hy- 
dration of hydrophobic groups, electrostatics, hydrogen
bonding, and conformational entropy (51). The two RST domains have similar folds and conformational stabilities but exhibit different unfolding $\Delta C_p$s. Since the AtRCD1–RST domain is more dynamic and malleable in the native state, it is possible that the core of AtRCD1–RST exposes more hydrophobic surface than AtTAF4–RST in the native state, resulting in the smaller $\Delta C_p$ upon denaturation. The increased malleability of the native state, together with larger temperature dependence of stability (Fig. 3A), would ensure functionality in a large range of environments and with a large number of different TF ligands, as in the RCD1–interactome (13). In contrast, based on the observation that the AtTAF4–RST stability was less temperature dependent (Fig. 3A) and that the CSPs of initial unfolding were smaller and more widely distributed (Fig. 5), it is possible that AtTAF4–RST confers more narrow specificity (Fig. 1B) associated with more specific functional roles (Fig. 6). HsTAF4–TAFH was the most stable of the three $\alpha$-hub domains. This could be due to the larger size (39 residues longer) with more folded residues resulting in a larger $\Delta f$ and a larger $m$ value and a corresponding lower flexibility and could analogously explain its fewer known interaction partners (Fig. 1B).

Specificity for AD–coactivator interactions remains an intriguing question, dominated by the acceptance of functional interchangeability of ADs and coactivators (3–5), although with recent suggestions of specificity in these types of interactions (10, 12). Here, we analyzed the ability of the $\alpha$-hub domains to bind AtDREB2A. AtDREB2A is a biological ligand of AtRCD1, and interactions between AtRCD1 and AtDREB2A negatively regulates AtDREB2A (27, 52). AtDREB2A bounds AtRCD1–RST with high affinity but binds also both AtTAF4–RST and HsTAF4–TAFH with affinities typical of $\alpha$-hub–TF interactions (13) (Table 2). However, whereas the AtRCD1–RST–DREB2A interaction was driven by enthalpy with a considerable entropic penalty, the interactions of AtDREB2A with the other $\alpha$-hub domains were driven by large favorable entropic contributions. This was especially pronounced for the interaction with AtTAF4–RST, for which the enthalpic contribution was very low and binding-induced folding reduced compared with the interaction with AtRCD1–RST (Fig. 6). Speculating, thermodynamics may be a route to distinguish biological ligands from nonspecific ligands. AtDREB2A, as a biological ligand of AtRCD1–RST, forms an extensive network of specific noncovalent bonds with AtRCD1–RST (13, 14), absent in complex with the two other $\alpha$-hubs. In these cases, retained flexibility rather than noncovalent bonds may drive the interactions through a reduced loss of conformational entropy (13, 14). In accordance with a general model for intrinsic disorder–based interactions (53), the disordered RCD1-binding SLiM of DREB2A would initially bind all three $\alpha$-hub domains in multiple different conformations, likely using interaction hot spot residues (27, 54). Then, only in complex with AtRCD1–RST, it would fold cooperatively with AtRCD1–RST into a native complex with extensive formation of specific noncovalent bonds as well as more helix stabilization in the hub itself (Fig. 6). In this case, changes in binding enthalpy govern high-affinity complex formation, potentially leading to longer lifetimes of the biologically relevant complexes. In other cases, entropy may also be important for formation of high-affinity complexes (55–57). Together, the results show how conformational flexibility of intrinsic disorder contributes to protein–protein interactions by allowing partner adaptation (16, 53) and how the balance between binding enthalpy and entropy may fine-tune affinity but more importantly, specificity of AD–coactivator interactions.

In contrast to AtDREB2A, ANAC013 does not fold when binding to AtRCD1–RST (27, 28). The high affinity of ANAC013 for AtRCD1–RST is still sustained by binding enthalpy (27) (Table 2), whereas the two orders of magnitude weaker complex of ANAC013 with AtTAF4–RST is based on favorable entropic contributions. This raises the questions of whether ANAC013, also regulated by interactions with AtRCD1 in plant stress responses (24, 25), is indeed an in vivo ligand of AtTAF4. The expression patterns of the AtDREB2A and the ANAC013 genes are similar and induced in response to various hormones and stressors (20), whereas the AtRCD1 and AtTAF4 genes are constitutively expressed (30, 58). Even though induced levels of the TFs may enable low-affinity interactions to take place in vivo, AtRCD1 is likely to outcompete AtTAF4 for TF interactions. If AtDREB2A and ANAC013 are not in vivo ligands of AtTAF4–RST, what are then the ligands? Based on functional similarities of Arabidopsis and human TAF4, AtTAF4–RST may also exert narrow selectivity in interactions (13).

In this study, we explored the properties within interactomes that could be relevant for selectivity in hubs. We
determined the structure of the AtTAF4–RST domain, which allowed comparison of αα-hub domains and their interactions. Although the AtRCD1 TF ligands ATDREB2A and ANAC013 bound to both TAF4 αα-hub domains, NMR and thermodynamic analyses suggested that only biologically relevant αα-hub–TF pairs have evolved to specificity (57). Moreover, unfolding thermodynamics suggested the existence of a common thermal unfolding state with similar properties in all three αα-hub domains, but with varying temperature sensitivity, suggesting variability in structural adaptability relevant to binding. Taken together, the results showed that not only the flexibility of the TFs ease αα-hub–based protein–protein interactions, but that malleability of the hub domains also contributes to specificity in complex formation, with structure, dynamics, and thermodynamics of binding constituting routes for impacting interactome size.

**Experimental procedures**

**Bioinformatics analysis**

The domain architectures of AtRCD1 (Q8RY59), AtTAF4 (AT5G43130), and HsTAF4 (000268) were as reported in the Pfam database (59). The interactomes were obtained from the IntAct Molecular Interaction Database (60) for experimentally verified interactions. Multiple sequence alignment of the AtTAF4–RST, AtRCD1–RST, and HsTAF4–TAFH4 domains were made in ClustalOmega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (61).

**Protein expression and purification**

DNA encoding the TAF4–RST180–254 domain of AtTAF4 (AT5G43130) (29, 62) was cloned into pET-11a (Novagen), and the resulting construct verified by sequencing (TAG Copenhagen). The vector was transformed into competent *Escherichia coli* BL21(DE3) cells (Novagen) and subsequently grown in LB medium containing 100 mg ml⁻¹ ampicillin at 37 °C under shaking at 150 rpm. Expression of protein was induced with 0.5 mM isopropyl β-d-thiogalactopyranoside at an absorbance of 0.6 to 0.8 at 600 nm. After 3.5 h, cells were harvested by centrifugation (5000 g for 20 min). The supernatant was loaded onto a 2 ml of TALON Metal Affinity resin column (Clontech) equilibrated in 20 mM Tris–HCl, pH 7.0, and 100 mM NaCl. After binding, the resin was washed with the same buffer, and protein was eluted by adding imidazole to 200 mM. Tobacco etch virus protease (produced as described in Ref. (27)) at 1:100 w/w ratio was added, and cleavage was performed overnight and the protein subsequently dialyzed against the purification buffer without imidazole and in the presence of 2 mM DTT and 0.5 mM EDTA. Fractions containing the recombinant protein were further purified on a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with phosphate buffer (20 mM Na₂HPO₄/NaH₂PO₄, pH 7.4, and 100 mM NaCl). The eluted protein was concentrated using Centricon concentrators (Merck-Millipore) with a 3 kDa cutoff and stored at 4 °C. Final samples were analyzed by SDS-PAGE and MALDI-TOF mass spectrometry. Protein concentrations were calculated using the theoretical absorption coefficients at 280 nm obtained from ProtParam at the EXPASY server.

RCD1–RST180–254 was expressed and purified following the protocol as described (14), and AtANAC013254–274 (AT1G32870) and AtDREB2A243–272 (AT5G05410) were expressed and purified as described by O’Shea et al. (27).

**SAXS**

All SAXS measurements were carried out at the PETRA III, P12 beamline (DESY Synchrotron), at a working energy of 10 keV. The sample-to-detector distance of the X-rays was 3 m, and the exposure time was optimized to reduce radiation damage (Table S1). Six different concentrations of AtTAF4–RST180–254 were measured (from 1.1 to 8.9 mg ml⁻¹) (Table S2). Data from the highest and lowest concentration samples were discarded because of variation in the derived parameters. The samples were in 20 mM Na₂HPO₄/NaH₂PO₄, pH 7.0, 100 mM NaCl, and 1 mM DTT. The data were calibrated using water at the same temperature and analyzed using the ATSAS program package (https://www.embl-hamburg.de/biosaxs/software.html) (63). The higher concentration (4.2 mg ml⁻¹) was used to generate *ab initio* models with DAMAVER and DAMMIF programs from the ATSAS suite (63). The models resulting from 20 independent DAMMIF runs were superimposed using the DAMAVER tool, and the average filtered envelope was superimposed with the NMR structures using SUPCOMB (part of the ATSAS package (63)).

**NMR spectroscopy**

NMR data were acquired at 25 °C in 20 mM Na₂HPO₄/NaH₂PO₄, pH 7.0, 100 mM NaCl, 10% (v/v) D₂O, 0.02% (w/v) NaN₃, and 0.7 mM 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) and protein as specified. All spectra used for resonance assignment were recorded on a sample containing 580 μM 13C,15N-labeled AtTAF4–RST. For backbone chemical shift assignment, a set of 1H,15N HSQC, HNCA, CBCA(CO)NH, HNCO, HN(CA)CO, and (H)N(CA)NNH spectra were recorded on a Bruker AVANCE 600 MHz (1H) spectrometer equipped with a cryogenic probe. Side-chain assignments were performed from 1H–13C HSQC, HCCH-TOCSY, and 15N TOCSY–HSQC spectra recorded on a Varian INOVA 800 MHz (1H) spectrometer with a room
temperature probe. \(^{15}\text{N}\) NOESY–HSQC and \(^{13}\text{C}\) NOESY–HSQC spectra were recorded using a mixing time of 150 ms on the Varian INOVA 800 MHz spectrometer. A set of \(^{1}H,^{15}\text{N}\) HSQC, HNCA, and CBCA(CO)NH spectra were recorded on a Bruker AVANCE 800 MHz \(^{(1)H}\) spectrometer equipped with a cryogenic probe on a sample containing 200 mM \(^{13}\text{C}\), \(^{15}\text{N}\)-labeled DREB2A \(243\text{–}272\) in complex with 300 mM \(\text{ArTAF4}–\text{RST}\). All triple resonance spectra, except NOESY spectra, were recorded with nonuniform sampling at 25% and were reconstructed with quantum multiple-valued decision diagrams (64). All spectra were processed with NMRPipe (https://spin.niddk.nih.gov/bax/software/NMRPipe) and analyzed in CcpNMR analysis (65, 66). Random coil chemical shifts for calculation of secondary \(^{13}\text{C}\) chemical shifts were predicted by the webserver available at www.bio.ku.dk/english/research/bms/sbinlab/randomchemicalshifts2 (67).

**Structure calculations**

Backbone dihedral angle restraints were calculated using TALOS+ and, distance restraints were obtained from \(^{15}\text{N}\)-NOESY–HSQC and aliphatic and aromatic \(^{13}\text{C}\)-NOESY–HSQC spectra (68, 69). NOESY peaks were picked manually, whereas automated assignment and initial structure calculations were performed by CYANA (http://www.cyana.org/wiki/index.php/Main_Page) (68). Structure refinement with implicit water solvation potential EFFx (Effective Energy Function for XPLOR-NIH) (70) was performed using XPLOR-NIH resulting in 200 structures, of which the 20 lowest energy structures without significant violations were chosen to represent \(\text{ArTAF4}–\text{RST}\). Quality and statistics for the structural ensemble were evaluated with PROCHECK-NMR (71).

**NMR relaxation**

\(R_1\), \(R_2\), and \(^{1}\text{H}–^{15}\text{N}\) NOE relaxation parameters were determined from spectra recorded on a Bruker AVANCE 750 MHz \(^{(1)H}\) spectrometer equipped with a cryogenic probe using standard Bruker pulse sequences. Spectra were recorded on a sample containing 480 \(\mu\)M \(^{15}\text{N}\)-labeled \(\text{ArTAF4}–\text{RST}\). Relaxation delays of 20, \((3 \times 60), 100, 200, 400, (3 \times 600), 800, \) and 1200 ms were used for \(R_1\) and 16.96, \((3 \times 33.92), 67.84, 101.76, (3 \times 135.68), 169.60, 203.52, \) and 237.44 ms for \(R_2\). A recycle delay of 2.5 s was used in both experiments. For \(^{1}\text{H}–^{15}\text{N}\) NOE, two spectra with and without presaturation were recorded in an interleaved manner and with a recycle delay of 5 s. Data analysis was performed in CcpNMR analysis.

**CD spectroscopy**

CD spectra were measured using a Jasco 810 spectropolarimeter equipped with a Peltier thermoregulation system. Far-UV CD spectra were recorded between 200 and 190 nm with 0.1 mg ml\(^{-1}\) of protein in 20 mM sodium phosphate buffer (\(\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4\) at pH 7.4 and 1 mm path length. The scanning speed was 20 nm min\(^{-1}\), with data pitch of 0.1 nm. Each spectrum was averaged over 10 scans, and the spectrum of buffer, recorded identically, was subtracted from the protein spectrum. Helicity was calculated from \(\theta_{222}\) as described (72). For thermal unfolding, the protein concentration was increased to 1 mg ml\(^{-1}\), and the samples were in a buffer of 20 mM \(\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4\), pH 7.4, 100 mM NaCl. The signal followed a fixed wavelength of 222 nm in the temperature range of 20 to 90 °C, with data pitch 1 °C and a temperature slope of 1 °C min\(^{-1}\). Spectra were also recorded in the presence of increasing urea concentrations from 0 to 8 M. The urea concentration was measured with a Pocket Refractometer (ATAGO Co). Chemical denaturation was monitored by measuring the ellipticity values at 222 nm. Signals above the maximum value of the high-tension voltage, as provided by the spectropolarimeter manufacturer (600 V), were disregarded. Chemical and thermal denaturation curves were fitted as described later.

**Fluorescence spectroscopy**

Measurements were performed on the Prometheus NT.48 system (Nanotemper Technologies). Protein samples of 60 \(\mu\)M in phosphate buffer (20 mM \(\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4\), pH 7.4, and 100 mM NaCl) and in the presence of different urea concentrations (from 0 to 8 M) were analyzed in Prometheus NT.48 Standard capillaries (Nanotemper Technologies).

**Stability studies**

To obtain the \(\Delta G_{DN}\) at 25 °C and the \(m\) values, the chemical denaturation results measured by CD were fitted to Equation 1:

\[
y_{(c)} = \frac{y_N(c) + y_D(c) \exp \left( \frac{\Delta G - m c}{RT} \right)}{1 + \exp \left( \frac{\Delta G - m c}{RT} \right)}
\]

where \(y_{(c)}\) is the optical property at \(c\) (M) of denaturant; \(y_N(c)\) and \(y_D(c)\) are the optical properties of the native and the denatured protein molecules at \(c\) (M), respectively, and \(R\) is the gas constant.

Thermal denaturation was analyzed using the nonlinear least square fitting:

\[
y(T) = \frac{y_N(T) + y_D(T) \exp \left( \frac{\Delta H_{\text{DN}} \left(1 - \frac{T}{T_m}\right)}{RT} \right)}{1 + \exp \left( \frac{\Delta H_{\text{DN}} \left(1 - \frac{T}{T_m}\right)}{RT} \right)}
\]

where \(y(T)\) is the optical property at \(T\) (K) of denaturant; \(y_N(T)\) and \(y_D(T)\) are the optical properties of the native and denatured protein molecules at \(T\) (K), respectively, and \(R\) is the gas constant. The midpoint of denaturation (\(T_m\)) and \(\Delta H_{\text{DN}}\) were calculated for each protein. For the stability studies using CD spectroscopy, the spectra were analyzed using GraphPad Prism 9.0 (GraphPad Software, Inc).
The curves obtained from the fluorescence experiments were fitted to a two-dimensional model based on a two-step denaturation using Equation 3:

\[
y(T, [x]) = \frac{y_N(T) + y_D(T)e^x}{1 + e^x} = \frac{\Delta H_m \left(1 - \frac{T}{T_m}\right) + \Delta C_p \left(T - T_m \cdot T \ln \left(\frac{T}{T_m}\right)\right) - [x](m+m_1T+m_2T^2)}{RT} + \Delta H_m \left(1 - \frac{T}{T_m}\right) + \Delta C_p \left(T - T_m \cdot T \ln \left(\frac{T}{T_m}\right)\right) - [x](m+m_1T+m_2T^2)
\]

Equation 3 represents the global fit that consider both thermal and chemical denaturation, where \(\Delta H_m\) is the enthalpy change at the \(T_m\), \(\Delta C_p\) is the heat capacity change, and \(m\), \(m_1\), and \(m_2\) describe the \(m\) value at changing of denaturant concentration.

\(y_N(T)\) and \(y_D(T)\) describe the pretransition baseline and the post-transition baseline, respectively:

\[
y_N(T) = a_N + b_N T + c_N T^2 \quad (4)
\]

\[
y_D(T) = a_D + b_D T + c_D T^2 \quad (5)
\]

where \(a_N\), \(b_N\), \(c_N\), \(a_D\), \(b_D\), and \(c_D\) are temperature-independent coefficients. Pretransition and post-transition baselines of the denaturation experiments followed by CD spectroscopy were included in the fit but omitted from Fig S3. These baselines may be caused by solvent effects on the far-UV CD signal of the domains in the folded (pretransition) or unfolded (post-transition) states, respectively.

Gibbs free-energy change of protein unfolding was estimated with Equation 3, with values of \(\Delta H_m\), \(\Delta C_p\), \(T_m\), and \(m\), \(m_1\), and \(m_2\).

The global analysis of temperature and solvent denaturation was performed according to Ref. (44).

The \(C_m\) value was determined by

\[
C_m = \frac{\Delta G}{m}. \quad (7)
\]

**NMR titration experiments**

The interaction between AtTAF4–RST and AtDREB2A243–272 was investigated through a series of \(^1H\),\(^15N\) HSQC spectra recorded on samples containing 100 \(\mu\)M AtTAF4–RST in 20 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\), pH 7.0, 100 mM NaCl, 10% (v/v) D\(_2\)O, 0.02% (w/v) NaN\(_3\), and 0.7 mM DSS buffer and varying concentrations of DREB2A243–272 from 0 to 200 \(\mu\)M. Amide chemical shift perturbations between free and bound states were quantified using the weighted Euclidean distance (73):

\[
\Delta \delta^{15}N, H^{N}(ppm) = \sqrt{\left(\Delta \delta_1^{15}H\right)^2 + (0.154 * \Delta \delta_2^{15}N)^2} \quad (8)
\]

**NMR temperature experiments**

The chemical shifts of AtTAF4–RST at different temperatures were investigated through a series of \(^1H\),\(^15N\) HSQC spectra recorded on samples containing 100 \(\mu\)M \(^{15}N\)-labeled AtTAF4–RST or AtRCD1–RST in 20 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\), pH 7.0, 100 mM NaCl, 10% (v/v) D\(_2\)O, 0.02% (w/v) NaN\(_3\), and 125 \(\mu\)M DSS buffer, at 25, 30, 35, 40, 45, 50, and 55 °C.

**ITC**

ITC experiments were performed in a MicroCal ITC\(_{200}\) microcalorimeter (GE Healthcare). Protein samples at the concentration of 27 \(\mu\)M in the sample cell and 277 \(\mu\)M in the syringe were dialyzed against 50 mM Hepes buffer, pH 7.4, and 100 mM NaCl. ITC data were analyzed using an Origin 7 software package (MicroCal) and fitting to a one set of sites binding model. At least two experiments were performed for each interaction.

**Data availability**

Chemical shifts and NOESY data for AtTAF4–RST have been deposited in the Biological Magnetic Resonance Bank, www.bmrbr.wisc.edu.org under ID code 34557. Atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org, under ID code 7AC1.

**Supporting information**—This article contains supporting information (38).
Acknowledgments—We thank the staff at beamline P12 at DESY for assistance with recording of the SAXS data, Dr Andreas Prestel at cOpenNMR for NMR assistance, Dr Kaare Teilm for valuable discussion on the global fitting of the denaturation data, Dr Charlotte O’Shea for help with protein purification, and Dr Christian Buch Parsbæk and Anders Lønstrup Hansen for discussions and feedback on the article. Vilumfonden is thanked for support for NMR equipment, and NMR spectra were also recorded at cOpenNMR, an infrastructure supported by the Novo Nordisk Foundation (grant no.: NNF18OC0032996).


Funding and additional information—This work was supported by the Novo Nordisk Foundation challenge grant REPIN, rethinking protein interactions (grant no.: NNF18OC0033926; to B. B. K. and K. S.). Support was also achieved from the Novo Nordisk Foundation (grant no.: NNF18OC0032996). cOpenNMR, an infrastructure supported by the Novo Nordisk Foundation (grant no.: NNF18OC0032996).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: AD, activation domain; ANAC, Arabidopsis thaliana NAM, ATAF1/2, and CUC2; ARCD1, Arabidopsis thaliana RCD1; CSP, chemical shift perturbation; DREB2A, dehydration-responsive element–binding protein 2A; DSS, 2,2′-dimethyl-2-silapentane-5-sulphonate; Hs, Homo sapiens; H5QC, heteronuclear single quantum coherence; ITC, isothermal titration calorimetry; RCD1, radical-induced cell death1; RST, RCD1, SRO, and TAF4; SAXS, small-angle X-ray scattering; SLIM, short linear motif; SRO, similar to RCD one; TAF4, transcription initiation factor TFIIID-subunit 4; TAFH, TATA-box–associated factor homology; TF, transcription factor.

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