

Coupled Folding and Binding of the Disordered Protein PUMA Does Not Require Particular Residual Structure

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S Supporting Information

ABSTRACT: Many cellular proteins are ‘disordered’ in isolation. A subset of these intrinsically disordered proteins (IDPs) can, upon binding another molecule, fold to a well-defined three-dimensional structure. In the structurally heterogeneous, unbound ensemble of these IDPs, conformations are likely to exist that, in part, resemble the final bound form. It has been suggested that these conformations, displaying ‘residual structure’, could be important for the mechanism of such coupled folding and binding reactions. PUMA, of the BCL-2 family, is an IDP in isolation but will form a single, contiguous α -helix upon binding the folded protein MCL-1. Using the helix-breaking residue proline, we systematically target each potential turn of helix of unbound PUMA and assess the binding to MCL-1 using time-resolved stopped-flow techniques. All proline-containing mutants bound, and although binding was weaker than the wild-type protein, association rate constants were largely unaffected. We conclude that population of particular residual structure, containing a specific helical turn, is neither required for the binding nor for fast association of PUMA and MCL-1.

Intrinsically disordered proteins are predicted to be widespread in biology and are particularly abundant among eukaryotes.^{1,2} One potential explanation for the abundance of IDPs is that their coupled folding and binding reactions may offer an alternative to, and may have advantages over, the docking of two already folded proteins. For instance, it has been suggested that their disordered nature may lead to enhanced association kinetics,^{3–5} and the requirement to fold may allow the strength of binding to be decoupled from the specificity.⁶ Many examples of coupled folding and binding can be found in the BCL-2 family of proteins, the regulators of apoptosis in metazoans.⁷ MCL-1 is a folded BCL-2 protein with an exposed groove on its surface.⁸ The BH3 motif of PUMA, largely unstructured in isolation, adopts a single, contiguous α -helix upon binding this groove (Figure 1A).⁹ We have previously shown that PUMA associates with MCL-1 rapidly, near diffusion-limited, and binds very tightly (sub nM K_d).⁹

IDPs that undergo coupled folding and binding are not necessarily random coils in the unbound state. In the disordered ensemble some conformations are likely to have elements of secondary and tertiary structure, and some conformations may even resemble the final bound form.¹⁰ These, perhaps lowly populated, conformations of the unbound IDP have been labeled ‘preformed structural elements’,^{11,12}

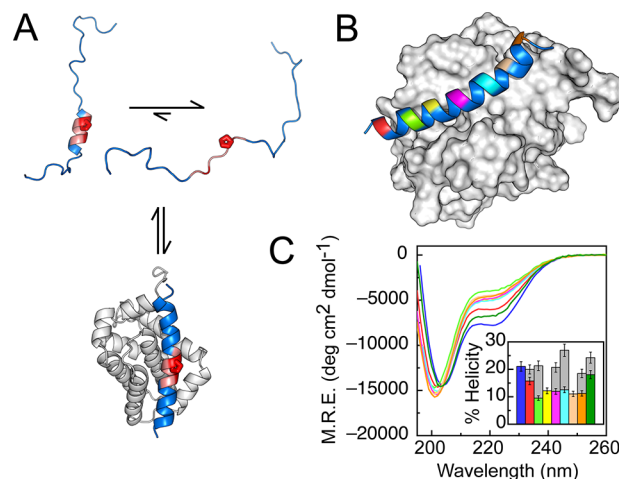


Figure 1. (A) Cartoon of the coupled folding and binding of PUMA IDP (blue) upon interaction with folded MCL-1 (white). The effect of proline on the unbound IDP is shown; helicity in the region of this residue is strongly disfavored. Bound structure is based on pdb 2ROC. (B) Cartoon of MCL-1 (white surface) with PUMA bound. Mutated residues; E132, red; E136, light green; A139, yellow; R143, magenta; D147, cyan; A150, beige; R154, orange; and E158 dark green, not present in structure. (C) CD spectra (mean residue ellipticity, MRE) of unbound PUMA IDPs. Inset shows calculated percent overall helical content for proline-containing mutant¹⁹ (color scheme identical to Figure 1B) and corresponding alanine mutant (gray).

‘intrinsically folded structural units’,¹³ ‘prestructured motifs’,¹⁴ ‘local structural elements’,¹⁵ and ‘residual structures’.¹⁶ There is intense effort, both experimentally and computationally, to characterize these conformations in unbound IDP ensembles, as they could be important for the association process.^{10,17} Indeed they are essential, according to the ‘conformational selection’ mechanism, where the interaction with the protein partner that leads to the final complex *only* occurs once the IDP has gained sufficient structure in isolation.¹⁸

Must an IDP display residual structure for association to occur? Are any specific residual structures more important in the association mechanism or even essential? Does the population of particular residual structures facilitate fast association kinetics? The PUMA MCL-1 system is well-suited to address these questions. The simplicity of the bound topology means that only residual α -helicity needs to be considered, and by using a peptide corresponding to the

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binding site, all residual helicity can be considered 'native'. The unbound PUMA IDP also has detectable residual structure to probe: the binding region of PUMA shows ~20% α -helical content by circular dichroism (CD).⁹

Fortunately, molecular biology affords us an exquisite, site-specific method to perturb residual helical structure—mutation to the residue proline.^{20–22} Proline is unique among the 20 naturally occurring amino acids, it is N-alkylated and cannot participate in i to $i - 4$ hydrogen bonding in α -helices. Furthermore, its cyclic chemical structure limits the torsional angles it and the preceding residue can adopt, often preventing the formation of the $i + 1$ to $i - 3$ H-bond.²³ This loss of two H-bonds results in proline being, by some margin, the most unfavorable amino acid to place in the center of an α -helix.^{24,25} In the context of an unbound IDP, and in the absence of significant tertiary interactions, mutation to proline will essentially abolish formation of the helical turn immediately N-terminal to the mutation (Figure 1A).

We carried out 'proline scanning' over the length of the IDP PUMA, individually targeting the eight turns of helix that could potentially form in the unbound state. To preserve the interface between the two proteins, the residues chosen for mutation—first to alanine and then to proline—were those of PUMA that remain solvent exposed when bound to MCL-1 (Figure 1B). Eight residues of wild-type PUMA were chosen for mutation, E132, E136, A139, R143, D147, A150, R154, and E158. To separate the loss of a charged wild-type residue from the introduction of proline, each position was additionally mutated to alanine.

Circular dichroism spectra provide a convenient measure of the overall helical content of a protein. All mutations to alanine led to either no changes in total helicity of PUMA or a small increase (Figure 1C, gray). In contrast, all proline-containing PUMA peptides had reduced overall helical structure relative to the wild-type peptide and the corresponding alanine mutant (Figure 1C, colored). This suggests that the residual helicity in the unbound wild-type peptide is not primarily located in one region but, rather, spread over its entire length. Mutation to proline led to only moderate reductions in total helicity, as due to the low cooperativity of helix formation,²⁶ the effect of proline is local and regions further away in sequence can presumably still occupy helical conformations. AGADIR¹⁹ helical predictions (Figure S1) demonstrate how a substitution for proline can cause a sharp drop in helicity local to the position of the mutation but only a moderate reduction of the global helicity. In general, the AGADIR predictions underestimated the experimentally measured total/global helical content (Table S1).²⁷

Surprisingly, although proline removes residual structure, removes hydrogen bonding, and introduces structural distortions,²³ every mutant underwent coupled folding and binding with MCL-1. CD spectra revealed that all proline-containing peptides bound to form a complex with greater helicity than the isolated components and with similar helicity to that of the wild-type complex (Figure S2). These data suggest that proline can be incorporated in the PUMA α -helix upon binding, however, in a similar manner to proline residues present at internal positions in the helices of folded proteins,²³ some distortion of the helix is likely.

Intrinsic tryptophan fluorescence has been used previously as a probe for binding, allowing the kinetics and thermodynamics of PUMA interacting with MCL-1 to be quantified.⁹ Here, all mutant PUMA bound MCL-1 with a similar change in

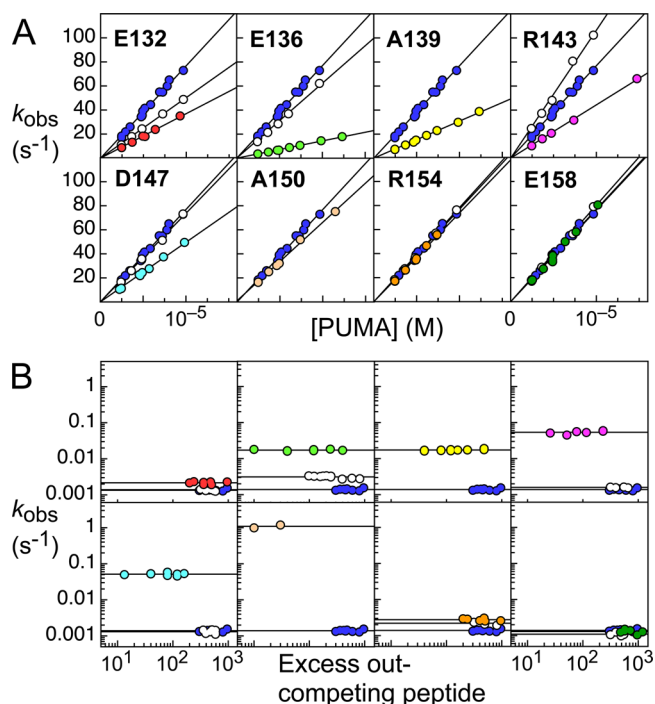


Figure 2. (A) Observed rate constants for the association of MCL-1 with wild-type and mutant PUMA peptides under pseudo-first-order conditions with PUMA in excess (slope corresponds to k_+). Rate constants for wild-type PUMA shown in white, alanine mutants in blue, and proline mutants in the Figure 1B color scheme. (B) Observed rate constants for the dissociation of wild-type and mutant PUMA peptides from MCL-1.

fluorescence to the wild-type, suggesting that a structurally similar complex is formed (SI results). Using stopped-flow kinetics and pseudo-first-order conditions, the association rate constants (k_+) were determined (Figure 2A, Table S1). By out-competition, using a peptide that shows no fluorescence change upon binding, the dissociation rate constants (k_-) were obtained (Figure 2B, Table S1). Equilibrium dissociation constants (K_d) were calculated from the ratio of the kinetic rate constants ($K_d = k_-/k_+$), as binding shows apparent two-state behavior (SI results).⁹ Mutations to proline were all destabilizing (Figure 3A, Table S1). The corresponding

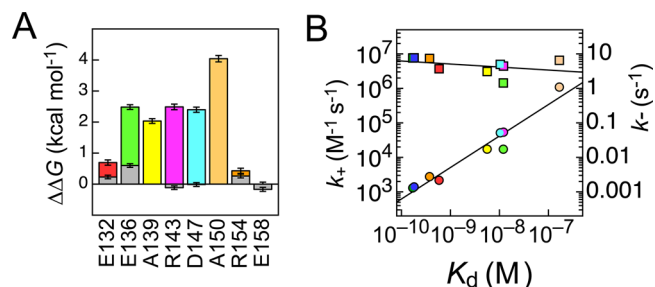


Figure 3. (A) Free energy of destabilization (for binding of MCL-1) caused by mutation of PUMA. Colored bars represent proline mutations, and gray bars represent the corresponding alanine mutation. Proline mutations are more destabilizing, especially toward the center of the binding region. (B) LFER plot of the kinetic and equilibrium constants for wild-type (blue) and the proline-containing PUMA (colored using Figure 1B scheme) binding MCL-1. k_+ shown as squares and k_- as circles.

mutations to alanine led to much smaller changes in stability, showing that the overall destabilization caused by proline mutation has little contribution from the loss of any charged surface residue. The free energy changes of binding, $\Delta\Delta G$, for the alanine to proline mutations ($<4 \text{ kcal mol}^{-1}$) are not larger than those reported for similar mutations at α -helical, solvent exposed sites in folding studies of model proteins,²⁴ and peptide association²⁵ reactions. This suggests that the protein–protein interface, and therefore the PUMA helix, is not significantly disrupted by these surface mutations.

Despite the presence of proline residues and the expected disruption of local residual helicity, all changes in k_+ were remarkably small in magnitude (Table S1). Unexpectedly, it was the increased dissociation rate constants, not the reduced association rate constants, which resulted in weaker binding. As shown by a linear free energy relationship (LFER) plot²⁸ $\log(k_+)$ varied little with $\log(K_d)$ (gradient -0.09 ± 0.08). Meanwhile, $\log(k_-)$ was essentially proportional to $\log(K_d)$ (gradient 0.91 ± 0.08) (Figure 3B). (Note that the effect of *cis*–*trans* peptide bond isomerization, which can complicate folding studies,²⁹ only had minor effects on the association kinetics and are discussed in SI results.)

Each individual proline mutation resulted in moderate (maximum 2-fold, Table S1) reduction in total residual helicity of the PUMA IDP and led to lower affinity for MCL-1. The link between binding affinity and population of residual structure has been established for other coupled folding and binding reactions.^{30–32} The kinetic basis for one such system was investigated recently³³ using structurally conservative mutations to perturb the residual helical content of the IDP, ACTR, with clear effects on both the association and dissociation rate constants for binding. We observe a similar, albeit weak, correlation between the total helical content of unbound PUMA and k_+ (Figure S6). Possibly the narrow range of total residual helicity and the nonconservative nature of proline mutations³³ mask a similar trend in this system, and there may be some small kinetic advantage from increased PUMA helical propensity. Alternatively, any differences between systems might result from differences in mechanism.

In this study, we take advantage of the fact that proline is extremely disruptive to local helix formation to investigate the requirement for particular helical regions for binding. All proline-containing peptides bind MCL-1. From this we can infer that no particular residual structure is essential for binding. Furthermore, fast association ($k_+ \sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$) does not require any individual turn of helix to be preformed in the unbound ensemble.

These results are inconsistent with a *pure* conformational selection mechanism, where the unbound PUMA IDP must exactly resemble the final bound form before productive collision with MCL-1. However, it is, in principle, possible that some degree of conformational selection could still occur whereby a shorter helical segment is selected by MCL-1, followed by induced fit of the remaining PUMA chain.^{34,35} The data presented here do not rule out a ‘mixed’ mechanism but show that no one particular helical region is essential for the proposed initial binding event. To explain the relative insensitivity of the association kinetics to the position of the proline mutations, there would need to be considerable redundancy in the helical structures that are capable of initiating binding. The large protein–protein interface and the long PUMA helix might facilitate this. Molecular recognition events with smaller interfaces and smaller elements

of folding secondary structure could be more dependent on specific residual structures. An alternative, and possibly more likely explanation for these results, is that residual helicity is not required at all, and association follows a induced fit mechanism.³⁶ As the global residual helicity is only moderately perturbed by these proline mutations, these two scenarios cannot be distinguished here. Indeed, flux through any of the above mechanisms could be occurring simultaneously and be dependent on the concentrations of the proteins involved.³⁷

This study reiterates¹⁰ that if a conformation resembling the bound structure can be detected in the unbound ensemble, this does not necessarily mean that this conformation is required in the mechanism of binding.

■ ASSOCIATED CONTENT

📄 Supporting Information

Supplementary results, table, methods, and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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