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**How robust is the mechanism of folding-upon-binding for an intrinsically  
disordered protein?**

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## **Abstract**

The mechanism of interaction of an intrinsically disordered protein (IDP) with its physiological partner is characterized by a disorder-to-order transition in which a recognition and a binding steps take place. Even if the mechanism is quite complex, IDPs tend to bind their partner in a cooperative manner, such that it is generally possible to detect experimentally only the disordered unbound state and the structured complex. The interaction between the disordered C-terminal domain of the measles virus nucleoprotein ( $N_{TAIL}$ ) and the X domain (XD) of the viral phosphoprotein (P) allows to detect and quantify the two distinct steps of the overall reaction. Here, we resort to analyze the robustness of the folding of  $N_{TAIL}$  upon binding to XD by measuring the effect on both the folding and binding steps of  $N_{TAIL}$  when the structure of XD is modified. Since it has been shown that wild-type XD is structurally heterogeneous, populating an on-pathway intermediate under native conditions, we investigated the binding to eleven different site-directed variants of  $N_{TAIL}$  of one particular variant of XD (I504A XD) that populates only the native state. Data reveal that the recognition and the folding steps are both affected by the structure of XD, indicating a highly malleable pathway. The experimental results are briefly discussed in the light of previous experiments on other IDPs.

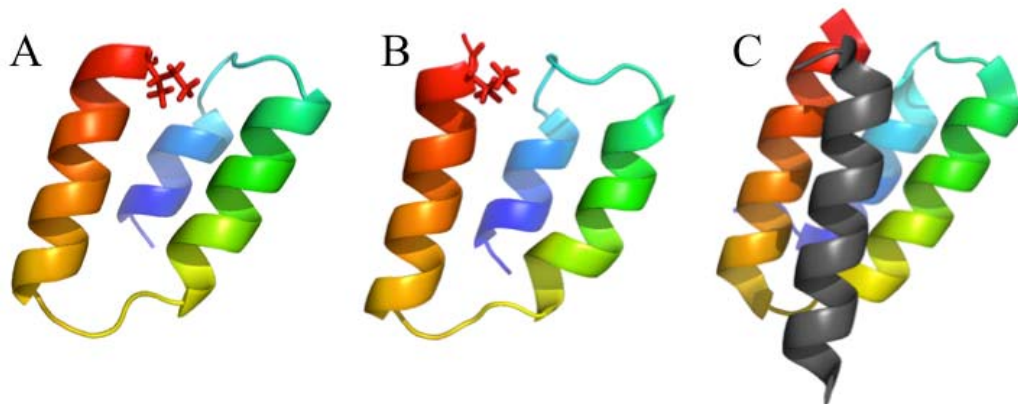
## INTRODUCTION

Intrinsically disordered proteins (IDPs) represent a class of fully functional molecules that lack a well-ordered structure in isolation under physiological conditions (1-7). A typical property of IDPs lies in their capability to undergo a disorder-to-order transition upon recognition and binding to their physiological ligand, a reaction that may lead to highly dynamic complexes, typically referred as “fuzzy” (8).

The folding-upon-binding reaction of IDPs is very complex since molecular recognition is intimately coupled to a structural transition. The binding induced folding of an IDP implies in theory at least two steps – the accumulation of the complex between the two interacting partners and the induced folding (9-11). These two steps might occur in different order, such that folding may precede (conformational selection) or follow (induced fit) binding. However, it has been previously reported that folding and binding tend to be co-operative, such that the overall reaction typically occurs in an all-or-none fashion and a single exponential decay is often observed in experimentally measured time courses (12-17). Such cooperativity is reminiscent of what is typically detected in the folding of globular proteins (18). In this context, the interaction between the intrinsically disordered C-terminal domain of the measles virus nucleoprotein ( $N_{TAIL}$ ) and the X domain (XD) of the viral phosphoprotein (P) is particularly interesting. In fact, in this case, the fortuitous complexity of the dependence of the observed rate constant upon (un)binding allows addressing quantitatively the two different steps of the overall process, by analyzing quantitatively the dependence of the relaxation rate constants (19-21). This feature represents a rare opportunity to interrogate directly the experimental system

concerning the nature and structural features of the folding and binding of an IDP, and possibly propose a generalization.

From a structural perspective, while  $N_{TAIL}$  is largely disordered (22), XD is a globular domain of 49 amino acids, consisting of a three-helix bundle (Figure 1) (23, 24). Upon binding to XD, the disordered stretch of amino acid residues 489-506 of  $N_{TAIL}$  acquires an  $\alpha$ -helical folding, the resulting structure of the complex corresponding to a four-helix bundle (Figure 1) (23-25, 26).



**Figure 1.** Cartoon representation of the three-dimensional structure of the free and bound forms of XD. A) Representation of the native structure of XD. B) Representation of the structure of the on-pathway folding intermediate of XD: it may be seen that while its secondary structure is native-like, its topology is somewhat different. C) Representation of the three-dimensional structure of the complex between native XD (in rainbow) and the residues 486-504 of  $N_{TAIL}$  (in gray) (PDB code: 1T6O) showing that the complex adopts a four-helix bundle conformation. Residue I504 is highlighted in sticks in figure A and B.

Previous investigations on XD in isolation have indicated that, while folded, this domain is structurally heterogeneous, populating an alternative state similar to an on-pathway folding intermediate (27). This state, while retaining a native-like secondary structure content, displays some significant differences in the overall topological organization (Figure 1). Importantly, the stability of the intermediate may be tuned by site-directed mutagenesis, such that variants populating solely the native state of XD were successfully obtained (27).

In this work we resorted to analyze the robustness of the folding of N<sub>TAIL</sub> upon binding to XD by analyzing individually the effect of any given perturbation of the structure of XD upon the folding and binding steps of N<sub>TAIL</sub>. In particular, our strategy was based on comparing the recognition of N<sub>TAIL</sub> to wild-type XD (characterized by a mixture of native and intermediate states) with a previously characterized variant of XD (I504A), that lacks the intermediate. The results rely on an extensive mutational study whereby we carried out kinetic experiments with 11 different site-directed variants of N<sub>TAIL</sub> challenged with the two forms of XD. Comparative analysis of the kinetic data shows that the binding and folding steps of N<sub>TAIL</sub> are both dictated by the structure of XD, indicating that the mechanism of recognition of this IDP is very malleable and appears to be sculpted by the topology of its physiological partner.

## **MATERIALS AND METHODS**

### *Expression and purification*

The variant I504A XD has been already described (27). All the variants of N<sub>TAIL</sub> have been produced by using the Quick-Change Lightning Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. Mutations were all confirmed by DNA sequencing. All proteins were expressed and purified as previously described (19, 20).

### *Temperature-jump experiments*

Binding experiments between I504A XD and the variants of N<sub>TAIL</sub> have been carried out by using a Hi-Tech PTJ-64 capacitor-discharge T-jump apparatus. A constant concentration of I504A XD (5 μM) was mixed with N<sub>TAIL</sub> wild-type and its variants at different concentrations, ranging from 2 to 40 μM, in 50 mM sodium phosphate buffer pH 7.2, with 300 mM NaCl. Carefully degassed samples were pumped through the 0.5x2 mm quartz flow cell before T-jump and data acquisition. Temperature was rapidly increased from 16°C to 25°C, with a temperature jump of 9°C. For each experimental condition, 10 traces were usually averaged and fitted to a single exponential equation. The excitation wavelength used was 296 nm and Trp fluorescence emission was collected using a 320 nm cut-off filter. Control experiments to calibrate the temperature jump were performed by measuring the changes in absorbance of a pH indicator (phenol red) in a buffer of known pH dependence (Tris-HCl) on temperature. Under the experimental condition explored, a discharge of 12 kV corresponded to an increase in temperature of 9 °C. Additionally, the fluorescence of N-acetyltryptophanamide (NATA) was used to monitor the time scale of the rapid heating. In fact, the quantum yield of this amino acid is highly dependent on temperature and represents a classical and simple tool to evaluate the

dead time of T-jump devices. Under the condition explored, heating was completed after about 20  $\mu$ s. Since the time scale of such an optical effect is much faster than that associated with the kinetics of binding between XD and NTAIL, data below 20  $\mu$ s (reporting on the pre-trigger and the heating phase) were excluded from the analysis of the kinetic data.

### *Data analysis*

The fluorescence time courses obtained for N<sub>TAIL</sub> and its site-directed variants were fitted by using a single exponential equation to obtain the observed rate constant for the relaxation time. Observed rate constants were plotted as a function of N<sub>TAIL</sub> concentration and fitted to the following hyperbolic function, arising from an induced-fit model:

$$k_{obs} = \frac{[N_{TAIL}]k_F}{[N_{TAIL}] + K'_D} + k_U$$

where  $k_F$  and  $k_U$  are the folding and unfolding rate constants of N<sub>TAIL</sub>, respectively, and  $K'_D$  is the dissociation constant of the complex.  $\Delta\Delta G_{K'}$  and  $\Delta\Delta G_{folding}$  have been calculated for each variant in the following way:

$$\Delta\Delta G_{K'} = RT \ln \frac{K'_D{}^{wt}}{K'_D{}^{mut}}$$

$$\Delta\Delta G_{folding} = RT \ln \frac{k_F^{wt} k_U^{mut}}{k_U^{wt} k_F^{mut}}$$

## **RESULTS AND DISCUSSION**

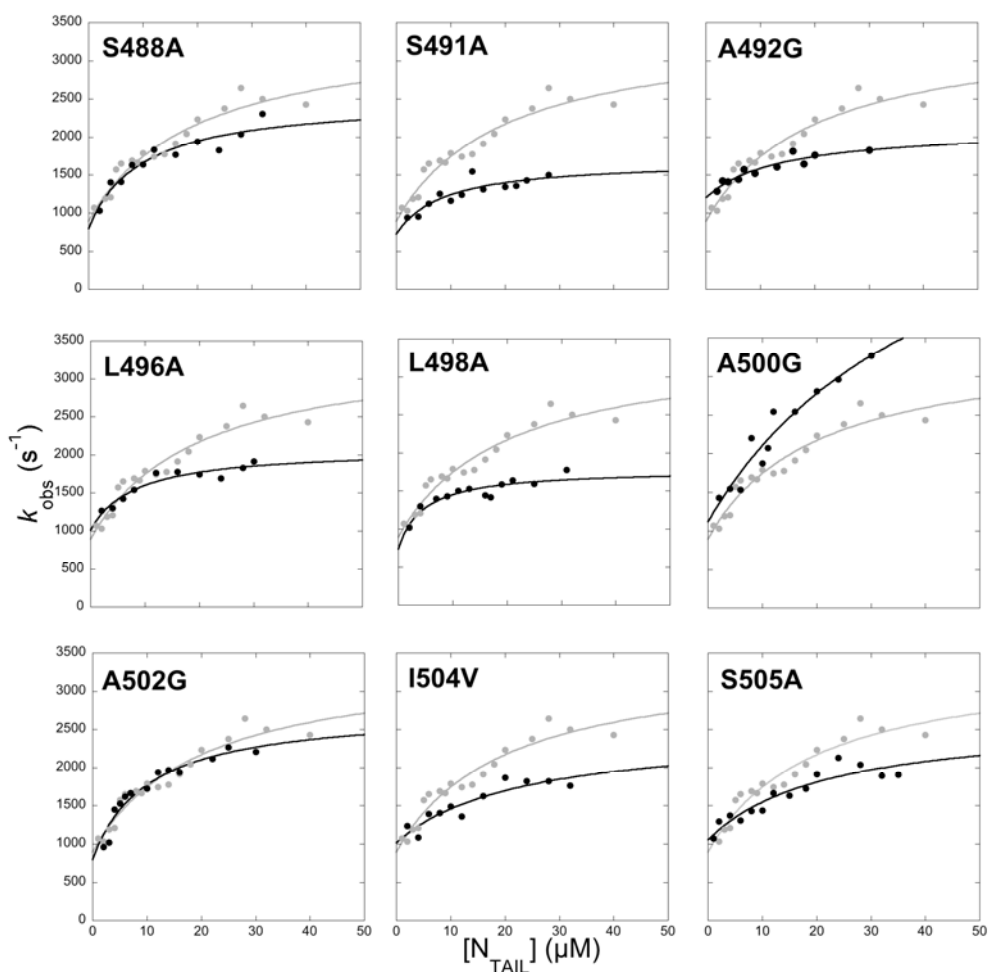


A classical method to elucidate the mechanism of a chemical reaction is to measure the effect of a perturbation imposed on the system on each detectable kinetic step (28-31). In the case of the binding reaction between  $N_{TAIL}$  and XD, we previously showed that these two proteins interact according to an induced-fit type mechanism, whereby the folding of  $N_{TAIL}$  is subsequent to the formation of an initial encounter complex (19, 20). In fact, by comparing the reaction kinetics when performing experiments in the presence of an excess of either  $N_{TAIL}$  or XD, we observed a superimposable hyperbolic dependence of the observed rate constants. This feature is a consequence of the symmetry of the reaction scheme of induced fit and is a signature that can be successfully used to exclude conformational selection (32, 33). Furthermore, we showed that both the binding and folding steps might be quantitatively addressed by analyzing the dependence of the apparent rate constant on reactant concentration (19, 20). Therefore, with the aim of analyzing the robustness of the folding and binding steps of an IDP, we used the variant I504A of XD, which populates solely the native state (27), and challenged this protein with eleven different site-directed variants of  $N_{TAIL}$ . The latter have all been recently prepared and employed to characterize the binding between  $N_{TAIL}$  and wild-type XD (20), which populates both the intermediate and native state.

Since the binding reaction is too fast to be followed by stopped-flow (19-21), we carried out the kinetic experiments using the temperature-jump methodology, incubating I504A XD at a constant concentration of 5  $\mu$ M with  $N_{TAIL}$  at different concentrations, typically ranging from 2 to 40  $\mu$ M. The relaxation process was triggered by a rapid increase in temperature of 9  $^{\circ}$ C, from 16 to 25  $^{\circ}$ C, using a

fluorescence-equipped capacitor-discharge temperature-jump instrument. In all cases, the time resolved fluorescence change, corresponding to an increase in emission, is consistent with a single exponential decay and a small perturbation of the equilibrium populations.

The dependence of the observed rate constant on the concentration of  $N_{\text{TAIL}}$  for each of the variant is depicted in Figure 2. In analogy to what we observed for wild-type XD (19-21), the profile of the concentration dependence is consistent for nearly all variants to a hyperbolic behavior, with the exception of A494G and L495A whose binding affinity was much too low to obtain reliable kinetics. Like the wild-type (19), all variants were fitted to an induced-fit mechanism as formalized in the Methods section; this allowed determination of the folding and unfolding rate constants as well as the overall affinity of the initial encounter complex, as expressed by its  $K'_D = k_{\text{off}}/k_{\text{on}}$ . The calculated parameters for all the variants are listed in Table 1.

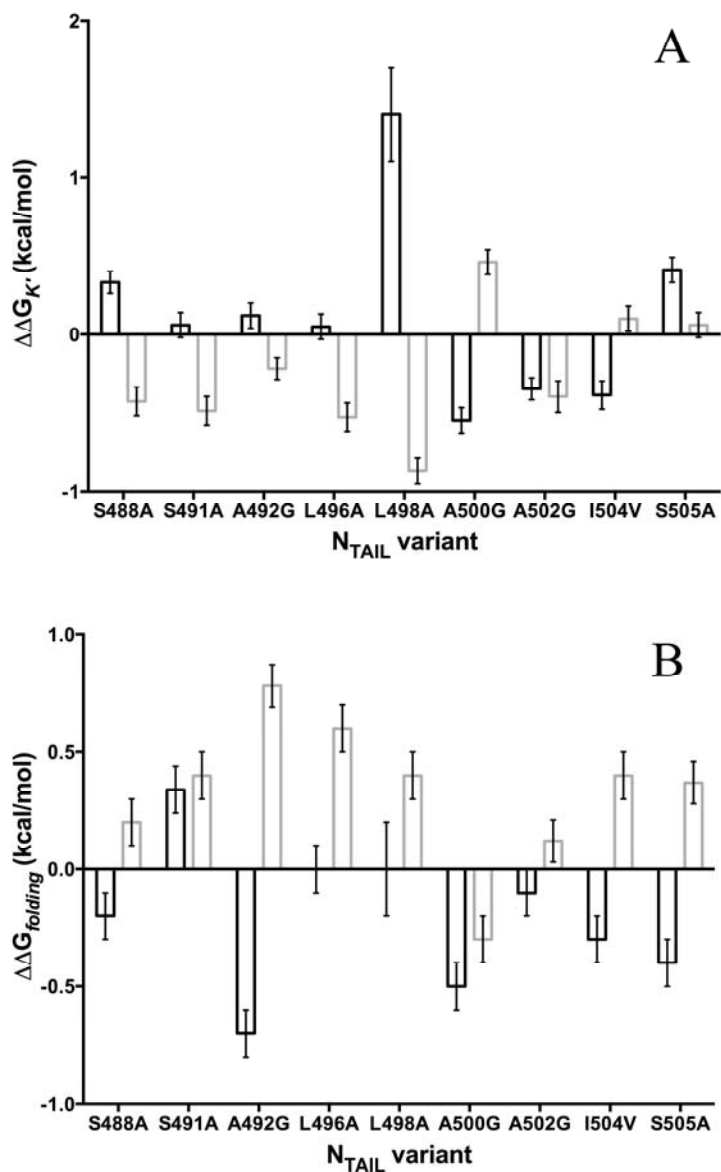


**Figure 2.** Binding kinetics of I504A XD and  $N_{TAIL}$  variants. The observed rate constants, measured by temperature-jump kinetics experiments, have been plotted as a function of the concentration of  $N_{TAIL}$ , typically ranging from 2 to 40  $\mu\text{M}$ . I504A XD was kept at a constant concentration of 5  $\mu\text{M}$ . In each plot, gray circles represent the kinetic binding experiments between I504A XD and  $N_{TAIL}$  wild-type, while black circles represent the kinetic binding experiments between I504A XD and site-directed variants of  $N_{TAIL}$ . For each variant, the overall dependence is consistent with a hyperbolic behavior. Variants A494G and L495A are not shown because their binding affinity was too low to obtain reliable kinetics.

VARIANT	$k_F$ (s <sup>-1</sup> )	$k_U$ (s <sup>-1</sup> )	$K'_D$ ( $\mu$ M)	$\Delta\Delta G_{K'}$ (kcal/mol)	$\Delta\Delta G_{folding}$ (kcal/mol)
WT	2500 $\pm$ 200	900 $\pm$ 60	20 $\pm$ 2	-	-
S488A	1700 $\pm$ 200	800 $\pm$ 100	10 $\pm$ 1	-0.43 $\pm$ 0.09	0.2 $\pm$ 0.1
S491A	1000 $\pm$ 100	720 $\pm$ 90	9 $\pm$ 1	-0.49 $\pm$ 0.09	0.4 $\pm$ 0.1
A492G	900 $\pm$ 100	1210 $\pm$ 50	14 $\pm$ 1	-0.22 $\pm$ 0.07	0.78 $\pm$ 0.09
L496A	1100 $\pm$ 100	1020 $\pm$ 70	8 $\pm$ 1	-0.53 $\pm$ 0.09	0.6 $\pm$ 0.1
L498A	1000 $\pm$ 100	700 $\pm$ 100	4.5 $\pm$ 0.4	-0.87 $\pm$ 0.08	0.4 $\pm$ 0.1
A500G	5200 $\pm$ 500	1100 $\pm$ 100	43 $\pm$ 4	0.46 $\pm$ 0.08	-0.3 $\pm$ 0.1
A502G	1800 $\pm$ 200	800 $\pm$ 80	10 $\pm$ 2	-0.4 $\pm$ 0.1	0.12 $\pm$ 0.09
I504V	1500 $\pm$ 200	1020 $\pm$ 80	23 $\pm$ 2	0.10 $\pm$ 0.08	0.4 $\pm$ 0.1
S505A	1600 $\pm$ 200	1050 $\pm$ 70	22 $\pm$ 2	0.06 $\pm$ 0.08	0.37 $\pm$ 0.09

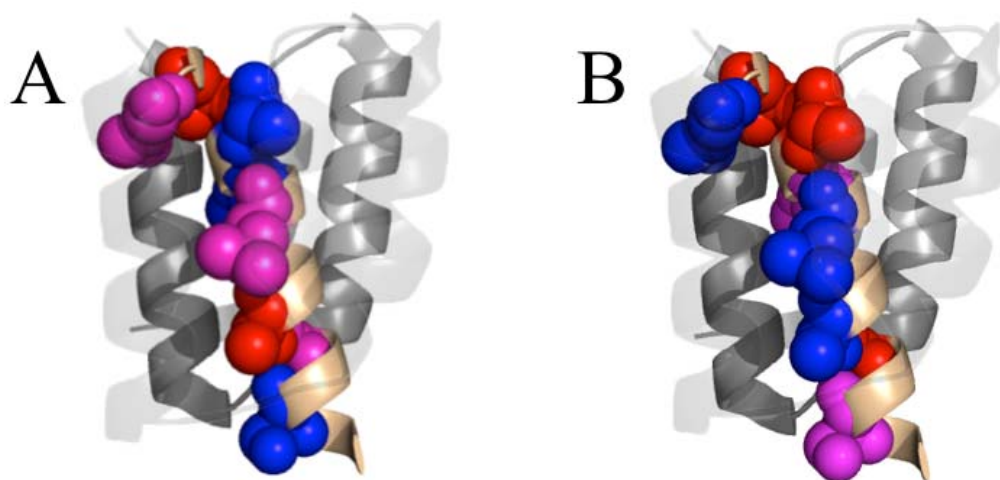
**Table 1.** Kinetic folding and binding parameters for the induced-fit reaction between I504A XD and N<sub>TAIL</sub> variants.

It is of interest to compare the effect of mutagenesis of N<sub>TAIL</sub> when this protein is challenged either with wild-type XD, populating both the native and intermediate states, or the I504A mutant, populating solely the native state. Figure 3 reports a bar chart representation of the calculated  $\Delta\Delta G$  for the folding and binding steps. It is evident that the recognition step, as mirrored by the  $\Delta\Delta G_{K'}$ , and the coupled folding step of N<sub>TAIL</sub> expressed by the  $\Delta\Delta G_{folding}$ , are both strongly affected by the 3D structure of XD, with some of the variants displaying a clearly different behavior as shown by comparing data obtained with wild-type XD and I504A XD. This finding suggests that both steps are influenced by the conformation of XD. Moreover, it should be noticed that, out of all eleven N<sub>TAIL</sub> variants studied, only L498A bears a substitution in a residue that is in direct contact with residue I504 of XD. Therefore this is the sole variant for which the observed changes in  $\Delta\Delta G_{K'}$  may result from direct contact between the side-chains.



**Figure 3:** Bar chart representation of the calculated  $\Delta\Delta G$ . For each variant of N<sub>TAIL</sub> we show the  $\Delta\Delta G$  calculated from experiments performed with XD wild-type (black bars) and with I504A XD (gray bars); each value being associated to its statistical error. A) Calculated  $\Delta\Delta G$  for the binding step ( $\Delta\Delta G_{K'}$ ). B) Calculated  $\Delta\Delta G$  for the folding step ( $\Delta\Delta G_{folding}$ ).  $\Delta\Delta G$  values obtained with XD wild-type were taken from (20)

In an effort to gain further insight on the effect of the conformation of XD on the folding and binding of  $N_{TAIL}$ , we calculated the difference between the  $\Delta\Delta G_{K'}$  obtained from kinetic experiments carried out with XD wild-type (populating two states) and that obtained in experiments performed with I504A XD. We also calculated in the same way the difference in  $\Delta\Delta G_{folding}$ . In this way, for each variant of  $N_{TAIL}$ , we obtained a  $\Delta\Delta\Delta G_{K'}$  and a  $\Delta\Delta\Delta G_{folding}$  (data shown in Table 1), which allowed us to highlight key changes on the structure of the complex (shown in Figure 4).



**Figure 4.** Cartoon representation of the  $\Delta\Delta\Delta G$  calculated for the complex between  $N_{TAIL}$  (gold) and XD I504A (gray), that populates only the native state. In the background the structure of the intermediate state of XD is shown. A) Calculated  $\Delta\Delta\Delta G_{K'}$  for complex formation. B) Calculated  $\Delta\Delta\Delta G_{folding}$  for  $N_{TAIL}$  folding. In each panel residues displaying  $\Delta\Delta\Delta G > 0.7$  kcal/mol are represented in blue; those displaying  $\Delta\Delta\Delta G$  between 0.4 and 0.7 kcal/mol are in magenta, and those with  $\Delta\Delta\Delta G < 0.4$  kcal/mol are in red.

In particular, a structural superposition between the intermediate, as obtained from restrained meta-dynamics using NMR chemical shifts (27), and the native states of XD reveal that the binding pocket for  $N_{TAIL}$ , located at the interface between helices 2 and 3, is clearly perturbed. Such a distortion appears to affect substantially the structure of the early recognition complex both in the C-terminal and in the N-terminal regions of  $N_{TAIL}$ , with residues displaying a high value of  $\Delta\Delta\Delta G_{K'}$  (shown in blue in figure 4) relative to both wild-type and I504A XD. It should be recalled that the high value of  $\Delta\Delta\Delta G_{K'}$  for L498A may result from a direct contact between this residue and position I504 of XD and may indicate that I504 may destabilize the binding of wild-type  $N_{TAIL}$  to the intermediate conformation. Interestingly, when folding is considered, it appears that the structural change within the partner and within the early encounter complex perturbs the folding of the whole helix, with clear changes in  $\Delta\Delta\Delta G_{folding}$  that extend to the central structural regions of the helix. Furthermore, the drastic change in the folding behavior of the A492G mutant, when challenged to the wild-type vs I504A XD, seems to suggest that the secondary structure of  $N_{TAIL}$  is destabilized when bound to the intermediate state of XD. A rigorous description of the binding mechanism would demand a complete analysis of the  $\Phi$  values, which is prevented by the relatively small changes in stability upon mutagenesis; however, we speculate that these experimental observations reflect what is expected in the case of heterogeneous nucleation, whereby the lack of a robust folding nucleus lead to a malleable mechanism, governed by the topological features of the binding partner (34, 35). In this case, both folding and binding of the IDP would be highly dependent on the physiological partner.

## CONCLUSIONS

The mechanism of recognition of many IDPs is a complex process involving at least two steps, the binding to the partner and the folding of the IDP (1-7, 9, 11). A comparison of the mechanistic features between the classical spontaneous folding of globular proteins with the binding induced folding of IDPs revealed some critical differences. In particular, while globular proteins fold via a robust mechanism, consolidated by the presence of a loosely formed yet specific nucleus (18, 36), IDPs appear to fold by heterogeneous nucleation, via an overall mechanism that is induced by interaction with the partner. In fact, an influence of the partner in the binding induced folding has been proposed by studying the interaction between PUMA and Mcl1 (14, 37) and later also observed for the interaction between KIX and c-Myb (35) and MLL (38).

Thanks to the possibility to distinguish between the folding and binding steps, the mechanism of the reaction between  $N_{\text{TAIL}}$  and XD unveiled in this paper allows to catch an additional glimpse of such a templated mechanism. In fact, mutational analysis of the binding and folding data reveals that both these steps are highly dependent on the fine shape of the physiological partner; this provides additional support to the notion that the energy landscape of IDPs retains a significant level of frustration in both the binding and folding steps, with a limited bias towards the main energetic minimum, even once bound to the partner. Future works on different, and structurally more complex, disordered systems will hopefully allow to test the generality of these conclusions.



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