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A folding-after-binding mechanism describes the recognition between the transactivation domain of c-Myb and the KIX domain of the CREB-binding protein

Stefano Gianni *, Angela Morrone, Rajanish Giri, Maurizio Brunori *

Istituto Pasteur-Fondazione Cenci Bolognetti and Istituto di Biologia e Patologia Molecolari del CNR, Dipartimento di Scienze Biochimiche "A. Rossi Fanelli", Sapienza Università di Roma, Piazzale A. Moro 5, 00185 Rome, Italy

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ABSTRACT

A large body of evidence suggests that a considerable fraction of the human proteome may be at least in part intrinsically unstructured. While disordered, intrinsically unstructured proteins are nevertheless functional and mediate many interactions. Despite their significant role in regulation, however, little is known about the molecular mechanism whereby intrinsically unstructured proteins exert their function. This basic problem is critical to establish the role, if any, of disorder in cellular systems. Here we present kinetic experiments supporting a mechanism of binding-induced-folding when the KIX domain of the CREB-binding protein binds the transactivation domain of c-Myb, an intrinsically unstructured domain. The high-resolution structure of this physiologically important complex was previously determined by NMR spectroscopy. Our data reveal that c-Myb recognizes KIX by first forming a weak encounter complex in a disordered conformation, which is subsequently locked-in by a folding step, i.e. binding precedes folding. On the basis of the pH dependence of the observed combination and dissociation rate constants we propose a plausible mechanism for complex formation. The implications of our results in the light of previous work on intrinsically unstructured systems are discussed.

1. Introduction

Much of our current understanding of proteins is based on the structure-function dogma, 'sequence determines structure determines function'. The fairly recent discovery that a significant fraction of proteins lacks a well-defined structure under native conditions and is intrinsically disordered (known as Intrinsically Denatured Proteins, IDPs) is set to revolutionize our understanding of protein structure–function relationships [1–7]. Despite their disordered structure, IDPs are often functionally competent being engaged in complex mechanisms of protein–protein or protein–DNA recognition. Moreover, IDPs when bound to targets undergo a structural transition to a folded state. The scientific community has been recently challenged to reconcile the unexpectedly high fraction of IDPs with the structure–function dogma. Why are some proteins disordered under native conditions, while others are folded? Is there any potential value for a protein to display a flexible structure that can be so extreme as to compromise the native state?

There are several hypotheses on the significance and value of disorder, which demand experimental validation [5]. From a thermodynamic viewpoint, it has been suggested that destabilization of a native fold may lower the affinity of a protein for its ligand, without necessarily compromising specificity [6,8]. Also, an interesting mechanistic model proposed by Wolynes and co-workers suggests the IDPs to display an increased capture radius to recruit and bind partners [9]. According to this view, called fly-casting mechanism, a disordered protein should form with its physiological partner a high-energy complex that would be locked in place by the coupled folding reaction. Whilst the kinetic data of binding and recognition of IDPs to their physiological partners are relatively scarce, it is of critical importance to understand the mechanisms whereby disordered systems operate [10,11].

The CREB-binding protein (CBP) is a co-activator that mediates the interaction between DNA-bound activator proteins and the components of the basal transcription complex. A well known globular domain of CBP, known as KIX, modulates such interactions [12]. Despite its small size, 87 amino acids, and a relatively simple fold, the KIX domain binds different IDP systems. Two different binding sites mediate such interactions, called c-Myb and MLL sites (named after two characteristic ligands of each of the site, i.e. the transactivation domain of the protein c-Myb and the mixed lineage leukemia MLL protein) [13]. Whilst the structural features of the interaction between KIX and different IDPs have been investigated by NMR spectroscopy in detail and several sets of co-ordinates are available in the pdb database [12–16], the

* Corresponding authors.
E-mail addresses: stefano.gianni@uniroma1.it (S. Gianni), maurizio.brunori@uniroma1.it (M. Brunori).

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mechanism of recognition is still a conundrum. More specifically, it is unclear whether KIX recognizes the IDPs in their ordered or disordered conformation – a critical question in tackling the proposition of an added value for disorder in IDP systems [10,17].

In this paper we present an extensive kinetic study to unveil the mechanism of binding and recognition between KIX and the transactivation domain of c-Myb, an IDP protein that folds into a helical structure once bound to KIX [15]. Our results clearly demonstrate that c-Myb recognizes KIX in an unstructured conformation and that binding precedes folding. Furthermore, by studying the binding kinetics as a function of pH, we propose a plausible recognition mechanism for complex formation.

2. Materials and methods

2.1. Site-directed mutagenesis and protein expression and purification

The site-directed mutant Y72W named pwtKIX was obtained by using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutation was confirmed by DNA sequencing. Proteins were purified by using a nickel(II)-charged chelating Sepharose FF (Amersham Biosciences) column equilibrated with 40 mM Tris–HCl and 500 mM NaCl, pH 8.5. The His-tagged wtKIX and pwtKIX were eluted with 250 mM imidazole. The samples were then diluted 4-fold in 40 mM Tris, pH 8.5 and all minor impurities were removed by the purification step on a Q-Sepharose column equilibrated with 40 mM Tris, pH 8.5. The proteins passed through the Q-column; the flow-through containing the protein was collected and concentrated. The purity of the protein was confirmed by SDS–PAGE.

The engineered construct indicated as c-Myb* where c-Myb was fused to the pro-domain of subtilisin was cloned into pPAL7 vector (BioRad). The protein was purified by using a cation-exchange chromatography (S-Sepharose column equilibrated with 40 mM Tris–HCl pH 8.5). c-Myb* was eluted with 850 mM NaCl. The sample was then diluted 4-fold in 40 mM Tris, pH 8.5 and all minor impurities were removed by the purification step on a nickel(II)-charged chelating Sepharose FF (Amersham Biosciences) column equilibrated with 40 mM Tris–HCl equilibrated pH 8.5. The protein passed through the nickel(II)-column; the flow-through containing the protein was collected and concentrated. The purity of the protein was confirmed by SDS–PAGE.

2.2. Equilibrium experiments

2.2.1. Circular dichroism (CD)

Far-UV CD spectra were recorded between 250 and 200 nm at a concentration of 10 μM using a Jasco spectropolarimeter (Jasco, Inc., Easton, MD, USA) and a 1-cm pathlength quartz cuvette (Hellma, Plainview, NY, USA). Data were fitted to a standard 2-state transition. The buffer used was 50 mM sodium phosphate pH 7.2.

2.2.2. Fluorescence

Equilibrium binding experiments were carried on a Fluoromax single photon counting spectrofluorometer (Jobin–Yvon, NJ, USA). Tryptophan fluorescence emission spectra were recorded in a cuvette (1 cm light path) between 300 and 400 nm. The excitation wavelength was 280 nm. pwtKIX concentration was typically 1 μM.

2.3. Stopped-flow measurements

Single mixing kinetic binding experiments were carried out on a SX-18 stopped-flow instruments (Applied Photophysics, Leatherhead, UK); the excitation wavelength was 280 nm and the fluorescence emission was measured using a 320 nm cut-off glass filter. The experiments were performed at 10 °C and the buffer used were: 50 mM Tris/HCl from pH 9.0 to 8.0, 50 mM sodium phosphate from pH 8.0 to 6.3, 50 mM sodium acetate from pH 5.5 to 3.8. All reagents were of analytical grade.

2.4. Temperature-jump fluorescence spectroscopy

The relaxation kinetics was measured as a function of [c-Myb] in absence or in presence of trifluoroethanol (TFE) by using a Hi-Tech PTJ-64 capacitor-discharge T-jump apparatus (Hi-Tech, Salisbury, UK). Temperature was rapidly changed from 16 to 25 °C with a jump-size of 9 °C. 10–20 individual traces were averaged at any given c-Myb concentrations. The fluorescence change of *N*-acetyltryptophanamide (NATA) was used in control measurements. Degassed and filtered samples were slowly pumped through the 0.5 × 2 mm quartz flow cell before data acquisition. The excitation wavelength was 296 nm and the fluorescence emission was measured using a 320 nm cut-off glass filter.

3. Results

To address the secondary structure of the transactivation domain of c-Myb in solution, we carried out equilibrium circular dichroism (CD) experiments at pH 7.2 and 25 °C. The far-UV CD spectrum of c-Myb, reported in Fig. 1, shows absence of secondary structure, confirming that c-Myb is an IDP [15].

The molecule 2,2,2-Trifluoroethanol (TFE), a well-known helix stabilizer, is known to induce folding in peptides and proteins [18,19]. Because c-Myb is helical once bound to its physiological partner KIX [15], we resorted to use TFE to induce folding in the free state. The CD spectra of c-Myb recorded in the presence of different concentrations of TFE (Fig. 1) show that c-Myb undergoes a folding transition with an apparent midpoint at 18% v/v TFE. The dependence of the CD signal at 222 nm upon [TFE] conforms to a standard two-state transition and allows calculating a folding free energy in water, $\Delta G^0 = 2.5 \pm 0.5$ kcal mol^{-1}.

3.1. Mechanism of binding of c-Myb to KIX

To study the mechanism of recognition between KIX and c-Myb, we produced different constructs characterized by a detectable fluorescence change upon binding. We first introduced a tryptophan residue in the proximity of the binding pocket and produced a pseudo-wild-type Y72W variant, called pwtKIX. The equilibrium binding transition of c-Myb to pwtKIX, monitored by fluorescence, is consistent with a simple hyperbolic behavior, with an apparent K_D of 20 ± 4 μM measured at pH 7.2 in the presence of 150 mM KCl and 25 °C (Fig. 1C).

We studied the kinetics of binding of c-Myb to pwtKIX using a temperature jump discharge capacitor apparatus (TGK Instruments, UK). A mixture containing a constant concentration of pwtKIX (10 μM), incubated with different concentrations of c-Myb, was subjected to a 9 °C rapid temperature jump, with a shift from 16 to 25 °C. Under all conditions, the observed relaxation kinetics were consistent with a single exponential time-course, suggesting the absence of detectable intermediates. The observed dependence of the relaxation rate constant on c-Myb concentration is reported in Fig. 2. Despite the inherent complexity of the folding-and-binding recognition reaction between pwtKIX and c-Myb, the observed relaxation behavior displays a simple second-order linear dependence. Furthermore, the apparent K_D of 30 ± 3 μM that can be calculated from the combination and dissociation rate constants is not inconsistent with the value obtained by equilibrium (K_D of 20 ± 4 μM), supporting the lack of detectable intermediates.

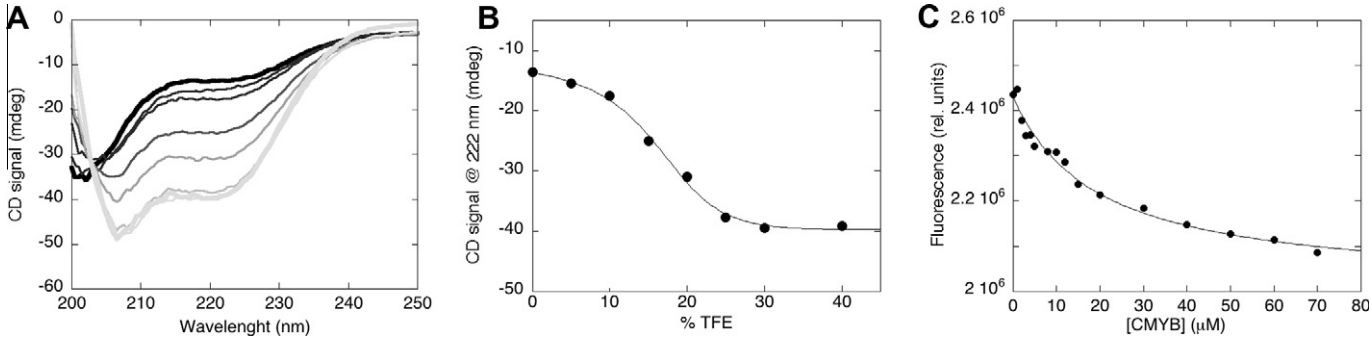


Fig. 1. (A) Far-UV CD spectra of c-Myb measured in 50 mM sodium phosphate buffer containing 150 mM KCl using a 1-cm cylindrical cuvette at pH 7.2 and 10 °C. [c-Myb] = 10 μM in the presence of TFE at increasing concentrations. From top to bottom spectra are depicted in a gray scale varying from black (in the absence of TFE) to light gray (in the presence of 40% TFE). (B) Dependence on [TFE] of the CD signal of c-Myb monitored at 222 nm in 50 mM sodium phosphate buffer, 150 mM KCl at pH 7.2 and 10 °C. Line is the best fit to a standard two-state transition. (C) Equilibrium binding transition of c-Myb to pwtKIX by fluorescence quenching, as observed in 50 mM sodium phosphate buffer, 150 mM KCl at pH 7.2. The emission wavelength was 370 nm (excitation 280 nm). pwtKIX concentration was 1 μM. Line is the best fit to a simple hyperbolic equation.

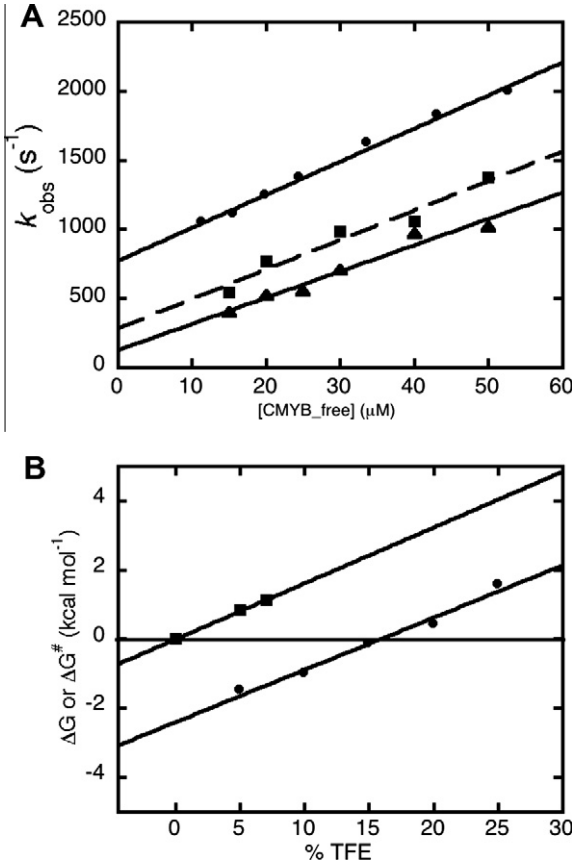
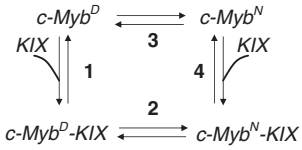


Fig. 2. (A) Dependence on the concentration of c-Myb of the relaxation rate constant measured by T-jump in 50 mM sodium phosphate buffer,150 mM KCl at pH 7.2, in the absence (●) and in the presence of 5% TFE (■) and 7% TFE (▲). Temperature was rapidly changed from 16 °C to 25 °C. (B) Dependence on [TFE] of the activation energy for dissociation of the c-Myb–pwtKIX complex (■), compared to the effect on the equilibrium free energy of folding of c-Myb (●), as calculated from the titration reported in Fig. 1.

To shed light on the function, if any, of disorder in IDP systems would require to clarify the order of events between folding and binding: does folding occur prior binding or do IDPs recognize their partner in a disordered conformation and fold only after binding? A monomeric protein undergoing a ligand induced conformational change can be described by a square mechanism (Scheme 1). Here c-Myb^D and c-Myb^N denote the denatured and folded



Scheme 1.

conformations of c-Myb, respectively. Complex formation progressing through pathway 1 and 2 is representative of an induced-fit model, whereby ligand binding induces a conformational change [20], i.e. folding-after-binding. Alternatively, binding progressing through pathway 3 and 4 assumes that two alternative conformations of c-Myb are in pre-equilibrium in the absence of the ligand, formally similar to a concerted model [21], or folding-before-binding.

To distinguish between the two different scenarios, we performed T-jump experiments in the presence of increasing concentrations of TFE, which stabilizes the helical conformation of c-Myb (Fig. 1). Remarkably, inspection of Fig. 2A reveals that, while the presence of TFE has a negligible effect on the apparent association rate constant, it stabilizes the c-Myb–KIX complex by lowering the dissociation rate constant. In fact, although experience suggests estimates of dissociation rate constants from pseudo-first-order dependences could be prone to large experimental errors, it is evident that, while TFE slows down binding, the slope of the second order plot is nearly unchanged. In analogy to the so called Φ-value analysis in protein folding studies [22], the lack of stabilization of the rate limiting step suggests the folding transition state to be as unstructured as the denatured c-Myb. Consequently, it may be concluded that folding of c-Myb is a late event occurring rapidly after the main rate-limiting barrier and being associated to the observed fluorescence quenching (Fig. 1), the overall reaction being consistent with a folding-after-binding mechanism. In these experiments, the highest concentration of TFE employed was 7% (v/v) because we found pwtKIX to precipitate at higher concentrations.

A powerful method to infer quantitatively the effect of a perturbation on the reaction mechanism is to compare the effect on activation free energy with that on equilibrium [23]. Fig. 2B depicts the dependence on the [TFE] of the activation energy for the dissociation of the c-Myb–KIX complex, compared to the effect on the equilibrium free energy of folding of c-Myb, as calculated from the TFE titration reported in Fig. 1. The linear dependence of the two parameters on [TFE] is essentially the same, while the association rate constant is essentially insensitive to [TFE] (Fig. 2A). This finding strongly suggests that the stabilization of c-Myb in its

helical folded conformation may account for all the stability the c-Myb–KIX complex. Furthermore, because the stabilization is due to a decrease of the dissociation rate constant, it may be concluded that the folding of c-Myb is a late step that occurs only downhill the main limiting barrier, i.e. c-Myb recognizes KIX in a disordered non-helical conformation.

3.2. Effect of pH on the binding kinetics

In order to produce c-Myb in high yields using *Escherichia coli* expression strains, we engineered a construct where the transactivation domain of c-Myb was fused to the pro-domain of subtilisin, hereby denoted as c-Myb*. While this construct binds to pwtKIX with an overall affinity comparable to that of free c-Myb, an investigation of its binding kinetics (Fig. S1) revealed a clear-cut effect on both the association and dissociation rate constants. These relatively limited changes are most likely due to some steric hindrance induced by the presence of the large tag, but do not affect the K_D for the binding to KIX. Furthermore, c-Myb* displayed the additional advantage of a much better signal to noise ratio compared to free c-Myb. Because of such improved signal to noise due to the tag, we resorted to expand the analysis of the mechanism of binding using c-Myb* and explored the effect of pH from 4 to 9.

The dependence of the observed rate constant on the concentration of c-Myb*, depicted in Fig. S2, shows that pH affects both the intercept and the slope, the overall behavior nevertheless being consistent with a simple bimolecular reaction. To validate the pH dependence of the apparent dissociation rate constant, we carried out a full set of displacement kinetic experiments where a pre-incubated complex between pwtKIX and c-Myb* was rapidly mixed with an excess of wild-type KIX (i.e. the wild type domain with a Tyr at position 72). Under appropriate conditions, the reaction is rate limited by the dissociation of the pre-existing complex, the excess of wild-type KIX substituting the bound pwtKIX in the complex with c-Myb*. The dissociation process was measured at different concentrations of wild-type KIX, ranging from 2- to 10-fold excess; the observed rate constants were found to be insensitive to wild-type KIX concentration at all pH values (Fig. S3). This methodology allows estimating unequivocally the dissociation rate constant, in analogy to classical experiments on myoglobin [24].

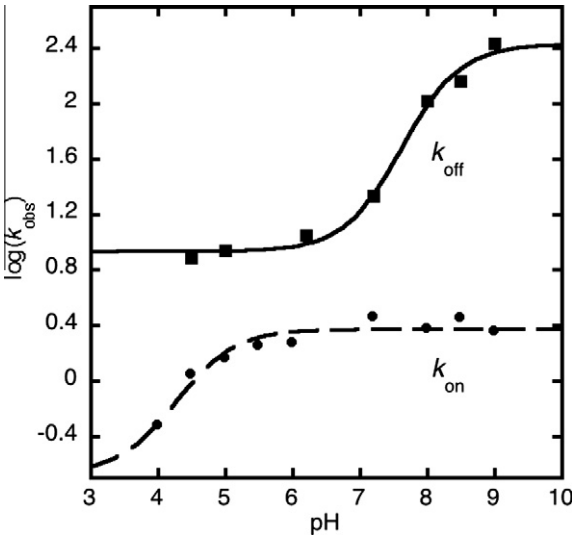


Fig. 3. Dependence of the logarithm of the apparent association (●) and dissociation (■) rate constants on pH. The apparent dissociation rate constants were obtained by a displacement experiment involving a rapid mixing of a pre-incubated complex between pwtKIX-c-Myb* with an excess of wtKIX, measured at 10 °C. Lines are the best fit to an equation implying a single protonation site.

A plot of the dependence of the apparent association and dissociation rate constants on pH is reported in Fig. 3. Both rate constants increase with pH following a sigmoidal profile, consistent with the protonation of a single group; the apparent pKa's for the association and dissociation rate constants are 4.2 and 7.6, respectively.

4. Discussion

The mechanism of recognition between IDPs and their physiological partners is expected to be a complex reaction that involves (i) the productive encounter between the two partners, (ii) the folding of the IDP system and (iii) the locking of key stabilizing interactions [1–7]. Despite the frequent occurrence of disorder in the proteome, its role is still shrouded in mystery, and even the order of these three key events in a folding-and-binding reaction is still unclear [10]. Hagen and co-workers [25] recently provided a complete characterization of the binding kinetics of the IDP protein IA3 to the yeast aspartic proteinase A; it was found that IA3 binds in a disordered conformation, a recognition event preceding folding. On the other hand, Onitsuka et al. [26] investigated the folding and binding reaction of disordered mutants of staphylococcal nuclease, which fold upon ligand binding. Interestingly, different variants seemed to follow either the folding-after-binding or the folding-before-binding mechanism, suggesting that the energetic partitioning between the different paths shown in Scheme 1 may be fine tuned via mutagenesis, as implicit in a squared model.

In a seminal work, Wright and co-workers [27] described the structural features of the interaction between the KIX domain and the IDP system pKID. Remarkably, by equilibrium NMR, it was found that partially folded species of pKID may bind to KIX, suggesting that, also in this case, the most plausible mechanism of IDP recognition implies a binding event preceding folding. Their equilibrium data, however, while providing the finest structural details of the interaction, could not unequivocally establish whether the recognition mechanism was a folding-after-binding rather than a folding-before-binding, a task that demands kinetic experiments. Identification of an intermediate by equilibrium measurements is not diagnostic on its role in the reaction mechanism, since it may be either on- or off- the productive pathway [28].

We report hereby an extensive kinetic characterization of the recognition reaction between KIX and c-Myb. Despite the inherent complexity of the process, it appears that, under all conditions explored, folding and binding are coupled and highly co-operative. Indeed no transient intermediates could be detected by fluorescence spectroscopy, the time course of the T-jump induced relaxation being described by a single exponential; and likewise the dissociation time course obtained by displacement was, under all conditions, consistent with a single exponential process. Comparison of the data recorded in the presence and absence of TFE, which stabilizes the helical conformation of c-Myb, sheds light on the order of events in the ligand induced folding. In fact, because TFE affects only the dissociation rate constant without influence on the association rate constant, it may be concluded that in the rate limiting step c-Myb is essentially unstructured. Therefore, binding precedes folding.

It is of interest to analyze the pH dependence of the observed association and dissociation rate constants (Fig. 3), whose pH dependence is consistent with the titration a single group with pKa of 4.2 and 7.6 respectively. Examination of the structure of the complex between folded c-Myb and KIX [15] (Fig. 4) reveals the presence at the interface of a single His residue on KIX (His17) forming a hydrogen bond with Thr305 of c-Myb, clearly compatible with the observed pKa of 7.6. Furthermore, two acidic residues at the interface, namely Glu306 and Glu308 of c-Myb

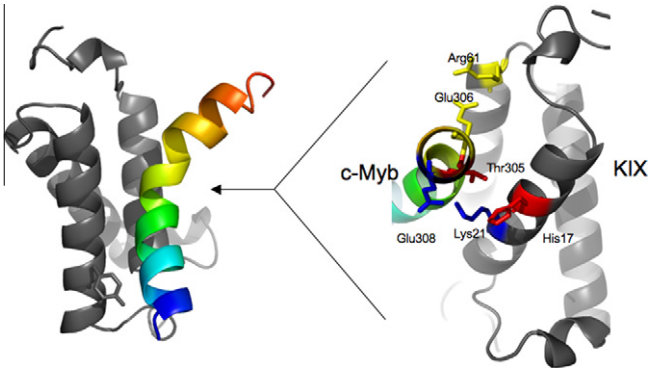


Fig. 4. Three dimensional structure of the complex between KIX and c-Myb, from the NMR data published by Wright and co-workers [15]. Residue Tyr72, mutated to Trp in pwtKIX, is highlighted in sticks on the structure on the left. The interaction surface, as seen from the right (see the arrow), is zoomed on the right of the figure. Amino acid side-chains highlighted are: His17-Thr305 (red); Lys21-Glu306 (blue); Arg61-Glu308 (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

form salt bridges with Arg61 and Lys21 of KIX, respectively. Both residues may be compatible with the pKa value of 4.2 controlling the association rate constant and could, therefore, be responsible for its pH dependence. We note, however, that while the Lys21-Glu308 salt bridge is adjacent to the His17-Thr305 interaction, the Arg61-Glu306 is located at the opposite side of the binding interface. Therefore, because the effects on the association and dissociation rate constants are distinct, we suggest Glu306 to be responsible for the pH dependence of the association rate constant. Overall, our data are consistent with a possible scenario whereby the rate limiting transition state is characterized by a largely unstructured conformation of c-Myb, which binds initially to KIX via a salt bridge between Arg61 and Glu306. The subsequent rapid folding step is driven by the locking interactions at the opposite side of the binding interface, involving the hydrogen bond between His17 and Thr305. In analogy with these observations, NMR relaxation dispersion on the KIX–pKID complex suggested the electrostatic interactions between His17 of KIX and Asp140/Asp144 of pKID to be formed at the latest stages of binding, being absent in the partially folded intermediate [27]. This finding would suggest the mechanism of recognition between KIX and different IDPs to display some conserved features. Future work based on Φ-value analysis will shed light on the atomistic details of the folding and binding reactions involved in the recognition and complex formation between KIX and its different IDP partners.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.112.

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