Unfolding of the C-Terminal Domain of the J-Protein Zuo1 Releases Autoinhibition and Activates Pdr1-Dependent Transcription

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Abstract

The C-terminal 69 residues of the J-protein Zuo1 are sufficient to activate Pdr1, a transcription factor involved in both pleiotropic drug resistance and growth control. Little is understood about the pathway of activation by this primarily ribosome associated Hsp40 co-chaperone. Here, we report that only the C-terminal 13 residues of Zuo1 are required for activation of Pdr1, with hydrophobic residues being critical for activity. Two-hybrid interaction experiments suggest that the interaction between this 13-residue Zuo1 peptide and Pdr1 is direct, analogous to the activation of Pdr1 by xenobiotics. However, simple dissociation of Zuo1 from the ribosome is not sufficient for induction of Pdr1 transcriptional activity, as the C-terminal 86 residues of Zuo1 fold into an autoinhibitory left-handed four-helix bundle. Hydrophobic residues critical for interaction with Pdr1 are sequestered within the structure of this C-terminal domain (CTD), necessitating unfolding for activation. Thus, although expression of the CTD does not result in activation, alterations that destabilize the structure cause induction of pleiotropic drug resistance. These destabilizing alterations also result in dissociation of the full-length protein from the ribosome. Thus, our results are consistent with an activation pathway in which unfolding of Zuo1’s C-terminal helical bundle domain results in ribosome dissociation followed by activation of Pdr1 via a direct interaction.

Legend: The 13 residues at the extreme C-terminus of the J-protein Zuo1 are sufficient for activation of the transcription factor Pdr1. Key hydrophobic residues are sequestered within an autoinhibitory four-helix bundle, necessitating unfolding of the C-terminal domain of Zuo1 for Pdr1 activation.
Introduction

The eukaryote-specific J-protein Zuo1 is an Hsp70 co-chaperone that is primarily associated with ribosomes and widely accepted to play an important role in the folding of nascent polypeptides. However, evidence from several organisms indicates that Zuo1 also has a direct role in transcriptional regulation. In Saccharomyces cerevisiae, Zuo1 has been identified as an activator of the zinc cluster transcription factor (TF) Pdr1. Zuo1 is a 433-amino-acid protein; however, the 69 residues at Zuo1’s C-terminus (Zuo1\textsubscript{365-433}) are sufficient to activate Pdr1-dependent transcription (Fig. 1a). The remainder of the protein contains regions, such as the J-domain, known to be important for its ribosome-associated role in protein folding. Particularly relevant to this report, the segment adjacent to the Pdr1-activating region is a positively charged RNA-binding region necessary for association with the ribosome.

Pdr1, like many TFs, has a C-terminal activation domain that has minimal activity until activated by a specific signal. Activation of Pdr1 by either Zuo1 or a variety of xenobiotics initiates a highly specific transcriptional response, upregulating a set of genes belonging to the pleiotropic drug resistance (PDR) regulon. Pdr1 target genes include ATP-binding cassette transporters, such as Pdr5 and Snq2. These transporters extrude xenobiotics from cells, rendering them resistant to a variety of toxic compounds. Evidence indicates that the PDR pathway also functions in growth regulation, perhaps by extruding small molecules sensed by neighboring cells.

Pdr1 is constitutively bound to DNA and not known to shuttle between the nucleus and cytosol. The C-terminus of Zuo1 interacts with Pdr1 in yeast two-hybrid assays, suggesting that Zuo1 activates Pdr1 directly. Since Zuo1 is primarily associated with the ribosome, this localization raises the question of the pathway of activation. Here, we report that the C-terminal domain (CTD) of Zuo1 forms an autoinhibitory four-helix bundle sequestering residues critical for Pdr1 activation. Unfolding of the CTD causes both dissociation of Zuo1 from the ribosome and release of autoinhibition necessary for PDR activation.

Results

Dissociation of Zuo1 from the ribosome is not sufficient for Pdr1 activation

We previously reported that a Zuo1 variant lacking the ribosome-binding region (Zuo1\textsubscript{Δ285–364}) was
competent to activate Pdr1, but full-length Zuo1 was not, even when overexpressed. This observation raised the question as to whether dissociation from the ribosome is sufficient for activation. Thus, we made constructs encoding variants having smaller deletions within the ribosome-binding region to test both ribosome association using sucrose gradient centrifugation and ability to induce PDR by plating on medium containing the drug cycloheximide. One variant, Zuo1Δ285–347, which has the same N-terminal deletion boundary as Zuo1Δ285–364 but retains 17 more residues at the C-terminal boundary, was not ribosome associated (Fig. 1b). However, cells expressing Zuo1Δ285–347 did not grow on drug-containing plates, while Zuo1Δ285–364-expressing cells grew as expected (Fig. 1c). We conclude that dissociation of Zuo1 from the ribosome is not sufficient to activate PDR.

Residues 348–364 inhibit the ability of Zuo1’s C-terminus to activate Pdr1

The only difference between the inactive Zuo1Δ285–347 and the active Zuo1Δ285–364 variants is the presence of 17 residues (348–364) in the former. Zuo1Δ365–433, the region immediately C-terminal to these 17 residues, is sufficient to activate Pdr1. Therefore, we reasoned that the inactivity of Zuo1Δ285–347 might be due to an inhibitory effect of these 17 residues. To test this idea, we created a construct encoding a tandem affinity purification (TAP)-tagged fusion analogous to TAP-Zuo1Δ365–433, which we previously reported to be competent to induce PDR, that included these 17 residues (TAP-Zuo1Δ348–433). Unlike cells expressing TAP-Zuo1Δ365–433, cells expressing the longer TAP-Zuo1Δ348–433 fusion showed no observable drug resistance (Fig. 2a and Supplementary Fig. 1a). We also assessed the ability of the TAP-Zuo1 fusions to activate the promoter of a Pdr1 target gene, PDR5, using a PDR5-lacZ fusion. Results were consistent with the drug resistance test. Cells expressing TAP-Zuo1Δ365–433 had 3.2-fold higher levels of β-galactosidase than control cells expressing only the TAP tag, but the TAP-Zuo1Δ348–433 fusion showed no activity above this background control level (Fig. 2a). Since our results were consistent with an inhibitory role for residues 348–364, we also made an intermediate

![Unfolding of the C-Terminal Domain of Zuo1](image-url)
construct that encoded TAP-Zuo1\textsubscript{358–433}. TAP-Zuo1\textsubscript{358–433}-expressing cells grew in the presence of cycloheximide but more slowly than those expressing TAP-Zuo1\textsubscript{365–433} (Fig. 2a and Supplementary Fig. 1a), suggesting partial inhibition of activity. Expression from the PDR5 promoter was also intermediate, with TAP-Zuo1\textsubscript{358–433}-expressing cells showing 2.5-fold activation compared to the 3.2-fold activation observed with the shorter fragment. These data indicate that residues 348–364 prevent activation of Pdr1 by the C-terminus of Zuo1. Thus, sequences sufficient for both transcriptional activation and inhibition of activity are contained within the last 86 residues of Zuo1.

Inactive and active C-terminal fragments differ in both stability and fold

To better understand the autoinhibitory effect of residues 348–364, we initiated a biochemical characterization of the Zuo1 C-terminal fragments. We were unable to obtain sufficient quantities of Zuo1\textsubscript{365–433}. Therefore, we compared the partially active Zuo1\textsubscript{358–433} and the inactive Zuo1\textsubscript{348–433} fragments. The melting temperatures of Zuo1\textsubscript{348–433} and Zuo1\textsubscript{358–433}, determined using circular dichroism (CD), were substantially different, 43.5 °C and 35.5 °C, respectively (Fig. 2b). Thus, removal of N-terminal residues, which resulted in partial activity, also resulted in a decrease in thermal stability. Analysis of the \(^{15}\text{N}–\text{H}\) heteronuclear single quantum coherence (HSQC) NMR spectra of Zuo1\textsubscript{348–433} revealed chemical shift dispersion and uniform peak intensity consistent with a single folded domain (Fig. 2c). The \(^{15}\text{N}–\text{H}\) HSQC of the Zuo1\textsubscript{358–433} sample, on the other hand, contained approximately twice the number of expected peaks, suggesting the presence of multiple structural populations. These data indicate that C-terminal fragments of Zuo1 that differ in their in vivo activity also differ in both stability and fold, suggesting the possibility that a structural transition in Zuo1’s C-terminus is responsible for activation of the protein’s transcriptional activity.

Autoinhibited C-terminus is a four-helix bundle

To understand the structural basis for this autoinhibition, we determined the solution structure of Zuo1\textsubscript{348–433} using an automated procedure for iterative nuclear Overhauser enhancement (NOE) assignment (Table 1). Zuo1\textsubscript{348–433} folds into a left-handed four-helix bundle (Fig. 2d and Supplementary Fig. 1b). To assure that sequences immediately N-terminal do not substantially affect the structure of the helical bundle, we generated two longer constructs extended by 13 or 32 residues. An overlay of the \(^{15}\text{N}–\text{H}\) HSQC spectra of Zuo1\textsubscript{335–433} and Zuo1\textsubscript{306–433} with that of Zuo1\textsubscript{348–433} revealed the addition of only random-coil peaks and showed no significant chemical shift perturbations between these fragments (Supplementary Fig. 1c), suggesting that the four-helix bundle formed by Zuo1\textsubscript{348–433}, which we refer to as the CTD, forms whether or not adjacent N-terminal residues are present.

Residues 348–364, which we identified as being inhibitory to Zuo1’s transcriptional activation activity, form the first helix of the bundle (Fig. 2d, green). Ile358, the N-terminal residue of the partially active Zuo1\textsubscript{358–433} fragment, is located in the center of helix I and buried within the structure. Comparison of the \(^{15}\text{N}–\text{H}\) HSQC spectrum of Zuo1\textsubscript{358–433} with that of the inactive CTD revealed a very similar pattern of dispersed peaks (Fig. 2c and Supplementary Fig. 1d), suggesting that the structured protein present in the partially active Zuo1\textsubscript{358–433} sample is similar in conformation to that of the inactive CTD. The additional peaks observed in the Zuo1\textsubscript{358–433} sample appear to be predominately clustered around the random-coil chemical shift value of −8.2 ppm, suggesting that a significant population of unfolded protein is present in the sample. These data are consistent with the idea that helix I is critical for stability...
Unfolding of Zuo1’s CTD releases autoinhibition

Our in vivo data indicate that Zuo1 is only fully active to induce PDR in the absence of helix I, which is integral to the CTD fold. Thus, we hypothesized that the C-terminus of Zuo1 activates Pdr1 in an unfolded conformation and that C-terminal sequences required to form the helical bundle structure cause autoinhibition. To test this idea, we designed two amino acid alterations aimed at destabilizing the fold of the domain: (1) the buried hydrophobic residue, Leu411, was replaced with the charged residue Arg and (2) Lys351 and Lys355 were replaced with prolines with the goal of preventing helix I from folding properly (Fig. 3a). We analyzed the $^{15}$N–$^1$H HSQC spectra of these variants. The spectra of both Zuo1348–433 L411R and Zuo1348–433 K351/355P showed poor peak dispersion with the majority of peaks clustered around the random-coil chemical shift value (Supplementary Fig. 2a), indicating that these amino acid alterations were sufficient to prevent folding of the CTD.

To test in vivo activity, we assessed the ability of TAP-Zuo1348–433 L411R and TAP-Zuo1348–433 K351/355P to induce PDR. Both were competent to render cells resistant to cycloheximide (Fig. 3b and Supplementary Fig. 2b). As a second in vivo test, we used a modified yeast two-hybrid assay that we described previously. In this assay, Zuo1365–433 was tethered to GAL1 promoters by expressing it as a fusion to the Gal4 DNA-binding domain (GBD). This fusion auto-activated transcription in a Pdr1-dependent manner, presumably by recruiting Pdr1, which in turn recruits RNA polymerase. Activity was assessed by growth on medium lacking histidine in a test strain containing a fusion between the GAL1 promoter and the HIS3 gene. We generated a GBD fusion construct analogous to GBD-Zuo1365–433 but containing the entire CTD, either having or lacking the L411R mutation that prevents folding. Cells expressing GBD-Zuo1348–433 did not form colonies (Fig. 3c) though expressed at expected levels (Supplementary Fig. 2c). However, cells expressing GBD-Zuo1348–433L411R grew as well as GBD-Zuo1365–433-expressing cells. We also tested a GBD fusion of Zuo1 residues 358–433, the fragment that had a mixture of folded and unfolded conformations in vitro. Cells expressing GBD-Zuo1358–433 formed colonies but grew more poorly than cells expressing the shorter GBD-Zuo1365–433 fusion (Fig. 3c and Supplementary Fig. 2c). Together, our data strongly support the idea that Zuo1’s C-terminus activates Pdr1 in an unfolded conformation.

Fig. 3. Unfolding of Zuo1’s C-terminal helical bundle releases autoinhibition. (a) Ribbon diagram of a portion of the CTD with side chains of residues predicted to disrupt domain structure upon substitution indicated. (b) We transformed wt cells with DNA encoding TAP tag fusions of the CTD with or without a destabilizing L411R or K351/355P alteration. Serial dilutions of cells containing the indicated plasmids were spotted onto medium without (−) or with (+) cycloheximide. (c) Modified yeast two-hybrid assay and correlation between protein fold and PDR induction. Cells containing an integrated GAL1-HIS3 reporter were transformed with DNA encoding the GBD alone (broken line) or GBD fused to fragments of the C-terminus of Zuo1, with the N-terminal residue of each fragment indicated. Transformants were spotted in serial dilutions onto medium with (+) or without (−) histidine. Table includes summarized PDR induction data from Fig. 2a and (b) with (++) representing a high level of induction and (−) representing no induction and summarized in vitro folding data from Fig. 2c and Supplementary Fig. 2a. Zuo1365–433 is predicted to be unfolded (see the text).
The C-terminal 13 residues of Zuo1 are necessary and sufficient for Pdr1 activation

While our results indicate that unfolding of Zuo1’s CTD is required for activation of Pdr1 and that the C-terminal 69 residues (365–433) are sufficient for this activity, the residues necessary for activity were not known. Thus, we constructed a series of truncations to generate TAP tag fusions beginning at residues 388, 403 and 421, but all ending at C-terminal residue 433, to test whether a smaller segment is sufficient. All shorter C-terminal fragments tested enabled cells to grow similarly to those expressing TAP-Zuo1365–433 on cycloheximide-containing plates (Fig. 4a). Also, β-galactosidase activities in cells expressing each of the shorter C-terminal truncations were statistically indistinguishable from the activity observed in TAP-Zuo1365–433-expressing cells, indicating an equivalent ability of the fusions to activate the PDR5 promoter. These results indicate that the C-terminal 13 residues of Zuo1 (421–433) are sufficient to activate Pdr1.

We carried out two additional experiments to determine the necessity of these 13 residues for activation. First, to confirm that these residues are necessary for activation in the context of the larger Zuo1365–433 fragment, we generated a construct that lacks the codons for these 13 residues, TAP-Zuo1365–420. Cells expressing TAP-Zuo1365–420 did not form colonies on cycloheximide-containing plates and had β-galactosidase activity similar to the basal level found in cells expressing only the TAP tag (Fig. 4a). Second, we constructed three additional TAP fusions having even smaller segments of the extreme C-terminus of Zuo1, generating TAP-Zuo1425–433, TAP-Zuo1428–433 and TAP-Zuo1430–433. None of these shorter C-terminal fusions were able to support growth on drug-containing plates (Fig. 4b and c). Thus, we conclude that the 13 extreme C-terminal residues (421–433) of Zuo1 are both necessary and sufficient for Pdr1 activation.

Residues critical for Pdr1 activation are buried in the C-terminal helical bundle

To identify individual residues required for Pdr1 activation within the 13-residue segment identified as sufficient, we performed alanine scanning mutagenesis. Cells expressing either wild-type (wt) TAP-Zuo1365–433 or TAP-Zuo1365–433 containing 1 of the 13 alanine substitutions were plated on medium containing or lacking cycloheximide. Cells expressing the Leu428, Leu429, Tyr431 or Val433 substitution variants did not grow on cycloheximide-containing plates (Fig. 5a and Supplementary Fig. 3).
Those expressing the Pro425 or Phe432 substitutions grew much more slowly than those expressing the wt fusion. Alteration of only one hydrophobic residue, Leu424, was tolerated, while alteration of the six other residues had no obvious effect on Pdr1 activation.

We also performed an analogous alanine scan using the modified two-hybrid assay. The pattern of effects of alterations in the GBD-Zuo1_365–433 fusions was very similar to that found with the TAP-Zuo1_365–433 fusions. Cells expressing GBD-Zuo1_365–433 in which any of the seven hydrophobic residues in the extreme C-terminus (Leu424, Pro425, Leu428, Leu429, Tyr431, Phe432 or Val433) were altered showed no growth on selective medium, indicating a failure to activate the Gal4-inducible reporters (Fig. 5a and Supplementary Fig. 3b). Alteration of any of the polar or charged residues, on the other hand, had no effect on activity. Thus, we conclude that hydrophobic residues within the extreme C-terminus play critical roles in PDR induction.

Examination of the position of these critical hydrophobic residues within the helical bundle structure revealed, as expected, that these residues are predominantly buried within the hydrophobic core of the domain (Fig. 5b). Thus, we hypothesized that residues required for PDR activation also play important roles in domain structure. To test this idea, we generated a construct to express the CTD of Zuo1 lacking the three most C-terminal residues (Tyr431, Phe432 and Val433), as these residues are not only important for activity but also deeply buried and particularly well constrained within the hydrophobic core of the domain (Fig. 5c). The $^{15}$N–$^1$H HSQC of this variant, Zuo1_348–430, showed poor peak dispersion with the majority of peaks centered around the random-coil chemical shift value, consistent with the protein being in an unfolded conformation (Fig. 5d), confirming the importance of these residues in maintaining domain structure.

Specificity of Zuo1’s hydrophobic C-terminus in Pdr1-dependent transcriptional activation

The sufficiency of such a short segment of Zuo1 for PDR activation, coupled with the importance of hydrophobic residues, raised the question as to the specificity of activation, as short hydrophobic...
peptides have been found to act as general transcriptional activators when tethered to DNA. Thus, we compared the transcription activation potential of the Zuo1 peptide with two other hydrophobic peptides, referred to as P201 (YLLPTCIP) and P223 (YLLPFLPY), which were originally selected for their ability to activate transcription from the GAL1 promoter when fused to GBD.19 Unlike cells expressing only GBD, cells expressing any one of the three peptide fusions, GBD-Zuo1421–433, GBD-P201 and GBD-P223, grew on plates lacking histidine, indicating activation of the GAL1-HIS3 reporter (Fig. 6, left). We next tested the ability of the fusions to activate transcription in the absence of Pdr1. As expected, Δpdr1 cells expressing GBD-Zuo1421–433 did not grow in the absence of histidine. GBD-P201 and GBD-P223, on the other hand, activated GAL1-HIS3 even in the absence of Pdr1.

As a second test of specificity, we asked whether cells expressing TAP tag fusions of peptides P201 and P223 could, like Zuo1421–433, activate PDR when not tethered to DNA. Unlike cells expressing TAP-Zuo1421–433, no detectable drug resistance was observed for cells expressing TAP-P201 or TAP-P223, whether or not Pdr1 was present (Fig. 6, right; Supplementary Fig. 4). Together, these data indicate that, although activation of Pdr1 by Zuo1 requires a short hydrophobic peptide, the observed transcriptional activation is distinct from the general transcriptional properties observed previously for hydrophobic peptides tethered to DNA.

C-terminal unfolding results in dissociation of Zuo1 from the ribosome

The results discussed above indicate that both dissociation of Zuo1 from the ribosome and unfolding of the CTD are necessary for the specific activation of Pdr1. To assess the effect of unfolding of the CTD on ribosome association, we tested the migration in sucrose gradients of two Zuo1 variants having amino acid alterations that cause unfolding of the CTD, Zuo1L411R and Zuo1Δ431–433. Unlike wt Zuo1, neither variant co-migrated with ribosomes during centrifugation (Fig. 7). Rather, both remained at the top of the gradient. The lack of ribosome association of these Zuo1 variants was consistent with the results discussed above.
variants is consistent with a folded CTD being required for association of Zuo1 with the ribosome.

Discussion

The data presented here indicate that unfolding of the extreme CTD of Zuo1 results in both its dissociation from the ribosome and release of autoinhibition unleashing its ability to specifically activate the Pdr1 TF. This dual effect of unfolding of the CTD suggests a pathway of communication between the cytosolic translational apparatus and the nuclear transcriptional machinery.

The extreme C-terminal 13 residues of Zuo1 are necessary and sufficient for activation of Pdr1, with hydrophobic residues playing critical roles in this activation. Although short hydrophobic peptides have been identified as recruiters of the general transcription machinery,\(^6,20,21\) Zuo1's C-terminus activates transcription specifically through Pdr1. This specificity is consistent with previously reported microarray data demonstrating that the PDR regulon is the major class of genes upregulated by the C-terminus of Zuo1.\(^6\) Positive two-hybrid interaction analyses indicate that the interaction between Zuo1 and Pdr1 is direct,\(^6\) pointing to a model in which Zuo1's C-terminal hydrophobic peptide interacts directly with Pdr1, leading to mobilization of Pdr1's activation domain that is thought to be inhibited by its central regulatory region.\(^6,22\) Such a mode of activation is analogous to the direct binding of xenobiotics observed for both yeast and mammalian TFs in PDR.\(^9,23,24\) It should be noted, however, that Pdr1 activation may be more complex, as Zuo1 forms a very stable heterodimer with the atypical Hsp70 Ssz1,\(^25\) often called the ribosome-associated complex. Ssz1, when not ribosome associated, also activates Pdr1.\(^26\) Thus, it is likely that Zuo1 and Ssz1 act in concert as a complex in the natural environment to activate Pdr1.

For Zuo1 to activate Pdr1, its C-terminus must be unfolded, as hydrophobic residues in the extreme C-terminus critical for activation of Pdr1 are sequenced within a four-helix bundle formed by the C-terminal 86 residues and are thus inaccessible for intermolecular interactions in the folded conformation. The positive correlation between unfolding and transcriptional activation we observed points to a scenario in which Zuo1's activity in Pdr1-dependent transcriptional activation is regulated by autoinhibition conferred by the CTD structure, with activity being induced upon a folding:unfolding transition of its helical bundle domain. Although, to our knowledge, there are few, if any, examples of unfolding as a requirement for a protein to activate a TF, unfolding as a mode of positive regulation certainly has precedents. Perhaps best understood is N-WASP, which, in its structured form, sequesters key hydrophobic residues in its C-terminal VCA region required for actin polymerization.\(^27\) Cdc42 binding releases this autoinhibition by disrupting the folded structure of a helical GTPase-binding domain. Unfolding of this domain releases the cofilin homology motif and allows the VCA region to activate the Arp2/3 complex.

Previous work identified an 80-residue charged region in Zuo1 (285–364) required for ribosome binding.\(^3,28\) The structural analysis reported here demonstrates that residues at the C-terminal boundary of this ribosome-binding region are necessary for formation of the C-terminal helical bundle. More experiments will be required to understand the molecular interactions responsible for Zuo1's association with the ribosome, both in terms of protein:RNA and protein:protein contacts, and the intramolecular effects of unfolding of the CTD. However, even without this information in hand, a plausible hypothesis is that the two requisite steps for Pdr1 activation, ribosome dissociation and unfolding of the CTD to expose critical hydrophobic residues, are coupled, as alterations that promote unfolding of Zuo1's CTD also result in dissociation of Zuo1 from the ribosome. Thus, a shift in the equilibrium of the CTD to an unfolded conformation likely increases the amount of soluble Zuo1 free to activate Pdr1.

The idea that Zuo1's CTD is active in an unfolded conformation raises the question of how the autoinhibited structure gains access to the unfolded state that results in both ribosome dissociation and exposure of residues required for Pdr1 activation. Depending on the free energy of folding (\(\Delta \text{G}_{\text{fold}}\)), a significant population of unfolded CTD may exist in equilibrium with the folded structure, as observed by two-dimensional (2D) NMR for the Zuo1\(^{358–433}\) construct (Fig. 2c). Based on the NMR spectra and CD measurements of thermal unfolding, we estimate that the Zuo1\(^{348–433}\) and Zuo1\(^{358–433}\) C-terminal fragments fold with relatively small margins of stability, with \(\Delta \text{G}_{\text{fold}}\approx –2 \text{ and } –1\text{kcal/mol, respectively. These estimates suggest that, in the absence of other stabilizing or destabilizing interactions, roughly 4% of the CTD would be unfolded, a percentage consistent with observations that the vast majority of Zuo1 is ribosome associated under typical laboratory conditions. When Zuo1 is bound to the ribosome and the majority of the CTD is folded, we predict that the Pdr1-activating epitope would be exposed at low levels by a basal level of equilibrium unfolding. Such a low amount of the unfolded, ribosome-free form is likely insufficient to induce Pdr1 activation. However, a binding partner that recognizes a distinct segment of the unfolded CTD would shift the equilibrium toward the unfolded state, with the net effect of destabilizing the autoinhibitory conformation and promoting Pdr1 activation.

In a broader physiological context, the relationship among Zuo1 (and Ssz1), the ribosome and cellular signaling is intriguing. The translational apparatus
has been repeatedly linked to growth control and cell cycle regulation. Indeed, activation of Pdr1 leads to premature growth arrest as cells transition from preferred to non-preferred carbon sources, presumably due to sensing by neighboring cells of metabolites exported by induced transporters. It is worth noting that transcriptional regulatory functions of ribosome-associated chaperones may extend beyond Zuo1::Ssz1. Components of the nascent chain-associated complex have been reported to function as transcriptional coactivators. The work presented here is a start in unraveling the complex regulatory pathway for ribosome-associated complex components. Further work will be necessary to understand the dynamics of the interaction of these chaperones with the ribosome, including the identification of factors that affect the folding:unfolding equilibrium of Zuo1’s CTD and the balance between their function in protein folding at the ribosome and their regulatory roles in extraribosomal transcriptional activation.

Materials and Methods

Yeast strains and plasmids

Yeast strains were isogenic with DS10 and contain the following mutations: his3-11,15 leu2-3,112 lys1 lys2 trp1Δ.Δ ura3-52. Δzuo1::HIS3 and Δpdr1::TRP1 have been described previously. A strain containing an integrated PDR5-lacZ reporter was created by digesting the pTH120 plasmid containing PDR5-lacZ::HIS3 with Stul and transforming the resulting fragment into DS10 wt or Δpdr1::TRP1 to direct integration at the PDR5 locus. TAP and TAP-Zuo1Δ365–433 plasmids were described previously. Additional plasmids created for this study are described in Supplementary Table 1.

Assays for PDR induction

To assay drug resistance, we subjected approximately 10 OD260 units of IEM503 containing the indicated plasmids (Promega) at a dilution of 1:1000. Yeast lysates were prepared by bead beating for 5 min and clarified by centrifugation at 14,000 rpm for 10 min. To fractionate polysomes, we applied approximately 10 OD260 units of lysate to the top of a 4-m1 tube containing a final gradient of 30% sucrose and centrifuged for 80 min at 4°C, and a SW50.1 Ti rotor (Beckman). Gradients were monitored for absorbance at 254 nm to detect monosomes and polysomes. Fractions were precipitated with 10% trichloroacetic acid, separated by SDS-PAGE and subjected to immunoblotting.

Modified yeast two-hybrid assay

Strains PJ69 and PJ69 Δpdr1::TRP1, the GBD and GBD-Zuo1Δ365–433 plasmids and yeast two-hybrid methods were described previously. Additional plasmids generated for this study are described in Supplementary Table 1. Modified yeast two-hybrid assay was carried out by detecting auto-activation of GAL1-HIS3 and/or Gal2-TRP1 reporters of PJ69 wt or Δpdr1 cells by monitoring growth on minimal medium lacking uracil for plasmid selection and either histidine and adenine or histidine and containing 2 mM 3-aminotriazol, as described previously. Plates were incubated for 2–3 days at 30°C before photographing.

Protein expression and purification

We expressed 8× His-tagged Zuo1 C-terminal fragments in Escherichia coli BL21[pREP4] cells from pQE308HT34, based plasmids described in Supplementary Table 1. Cells were grown at 37°C to an OD600 ≈ 0.8 in LB medium containing 150 µg/ml ampicillin and 50 µg/ml kanamycin; expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM, and cells were grown for an additional 3 h at 15°C following induction. Isotopically labeled proteins were prepared for NMR by growing cultures in M9 medium containing [15N] ammonium chloride and/or [13C] glucose as the sole nitrogen and carbon sources, respectively. Cells harvested from a 1-l culture were resuspended in 50 mM sodium phosphate (pH 7.4), 300 mM NaCl, 40 mM imidazole and 0.1% (w/v) 2-mercaptoethanol buffer containing an ethylenediaminetetraacetic-acid-free Complete Protease Inhibitor Cocktail tablet (Roche). Cells were lysed using a French pressure cell, and protein was purified by 4°C, and a SW50.1 Ti rotor (Beckman). Followed purification, the protein were concentrated to approximately 500 µl for analysis by NMR, and the purity and identity were verified by SDS-PAGE and/or mass spectrometry.

CD spectroscopy

Samples of Zuo1Δ348–433 and Zuo1Δ358–433 were prepared at a concentration of 20 µM in buffer containing 20 mM sodium phosphate (pH 6.5) and 50 mM NaCl. Thermal denaturation experiments were performed in a 1-mm cuvette, and the ellipticity was monitored at 222 nm over a temperature range of 10–70°C. Thermal denaturation curves were analyzed by

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nonlinear least-squares fitting as previously described to determine the melting temperature. \(^36\)

**NMR spectroscopy**

NMR samples were prepared in buffer containing 20 mM sodium phosphate (pH 6.5), 50 mM sodium chloride, 1 mM dithiothreitol and 5–10% \(^2\)H\(^2\)O. All 2D \(^1\)H–\(^1\)H HSQC spectra were acquired at 20 or 25 °C on a Bruker 500- or 600-MHz spectrometer equipped with a triple-resonance CryoProbe™ and processed with NMRPipe software. \(^37\) The Zuo1\(^{348-433}\) sample used for structure determination was prepared in the identical buffer at a concentration of 1.2 mM. All structural data were acquired at 10 °C using a field strength of 600 MHz. Backbone \(^1\)H, \(^15\)N and \(^13\)C chemical shift assignments for Zuo1\(^{348-433}\) were obtained automatically as previously described using peak lists from \(^1\)H, \(^15\)N–\(^1\)H HSQC, HNCO, HN(CO)CA, HN(CO)CACB, HNCA, HNCACB, HN(CA)CO and CO(CO)NH. \(^38\) Side-chain assignments were completed manually from three-dimensional HBBACONH, HCCONH, HCCH total correlation spectroscopy and \(^13\)C(aromatic)-edited NOE spectroscopy (NOESY)–HSQC spectra. Chemical shift assignments were >99% complete for Zuo1\(^{348-433}\). Heteronuclear NOE values were measured from an interleaved pair of 2D \(^1\)H–\(^1\)H sensitivity-enhanced correlation spectra recorded with and without a 5-s proton saturation period.

**Structure calculation and analysis**

The Zuo1\(^{348-433}\) structure was calculated using distance constraints obtained from three-dimensional \(^15\)N-edited NOESY–HSQC and \(^13\)C-edited NOESY–HSQC (\(t_{mix} = 80\) ms). Backbone \(\phi\) and \(\psi\) dihedral angle constraints were generated from secondary shifts of the \(^1\)H, \(^13\)C\(^\alpha\), \(^13\)C\(^\beta\), \(^13\)C and \(^1\)H\(^\beta\) nuclei using the program TALOS. \(^39\) Structure calculations were performed using the torsion angle dynamics program CYANA \(^40\) followed by iterative rounds of manual refinement to eliminate constraint violations. Of the 100 CYANA structures calculated, the 20 conformers with the lowest target function were subjected to a molecular dynamics protocol in explicit solvent \(^41\) using Xplor-NIH. \(^42\) PyMOL (Schrödinger, LLC) was used to generate all structure images.

**Accession numbers**

Coordinates and related data have been deposited at the Research Collaboratory for Structural Bioinformatics Protein Data Bank (2LWX); NMR data, at Biological Magnetic Resonance Bank (17685).

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**Supplementary Data**

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**Abbreviations used:**

PDR; pleiotropic drug resistance; TF, transcription factor; CTD, C-terminal domain; TAP, tandem affinity purification; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; GBD, Gal4 DNA-binding domain; wt, wild type; 2D, two-dimensional.

**References**


