Structure and Activity of the Peptidyl-Prolyl Isomerase Domain from the Histone Chaperone Fpr4 toward Histone H3 Proline Isomerization*

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Yoan R. Monneau‡, Hedy Soufari‡§, Christopher J. Nelson‡, and Cameron D. Mackereth‡

From the ‡Institut Européen de Chimie et Biologie, 2 rue Robert Escarpit, 33607 Pessac, France, the §INSERM U869, University of Bordeaux, 33076 Bordeaux, France, and the †Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia V8W 3P6, Canada

The FK506-binding protein (FKBP) family of peptidyl-prolyl isomerases (PPIases) is characterized by a common catalytic domain that binds to the inhibitors FK506 and rapamycin. As one of four FKBP5s within the yeast Saccharomyces cerevisiae, Fpr4 has been described as a histone chaperone, and is in addition implicated in epigenetic function in part due to its mediation of cis-trans conversion of proline residues within histone tails. To better understand the molecular details of this activity, we have determined the solution structure of the Fpr4 C-terminal PPIase domain by using NMR spectroscopy. This canonical FKBP domain actively increases the rate of isomerization of three decapetides derived from the N terminus of yeast histone H3, whereas maintaining intrinsic cis and trans populations. Observation of the uncatalyzed and Fpr4-catalyzed isomerization rates at equilibrium demonstrate Pro16 and Pro30 of histone H3 as the major proline targets of Fpr4, with little activity shown against Pro38. This alternate ranking of the three target prolines, as compared with affinity determination or the classical chymotrypsin-based fluorescent assay, reveals the mechanistic importance of substrate residues C-terminal to the peptidyl-prolyl bond.

Unique among the amino acid residues, proline can significantly populate both the usual trans backbone conformation as well as a cis arrangement in which the proline is flipped 180° with respect to the preceding residue. Although spontaneous, this isomerization event is slow, in some cases taking minutes to hours. Accordingly, prolyl isomerization can present a rate-limiting step to protein folding (1, 2). To alleviate this problem, the cell contains various peptidyl-prolyl isomerases (PPIases) to catalyze the isomerization rate of the peptidyl-prolyl bond and thus act as a protein folding chaperone (3). In addition, the structural bivalency of peptidyl proline on some proteins has been harnessed as a regulatory or signaling switch (4). Cyclophilins, parvulins, and FK506-binding proteins (FKBPs) comprise the three classes of PPIases found in all domains of life, and are defined predominantly on structural characteristics (5, 6).

The FKBPs share a canonical fold defined by the prototypic mammalian FKBP12. The overall architecture features a barrel shape defined by an extended ß-sheet and a single ß-helix, with a prominent solvent accessible hydrophobic pocket at one end. This pocket serves as the binding site of FKBPs, which recognize the peptidyl-prolyl moiety of the target polypeptide. However, despite the numerous isolated and inhibitor-bound FKBPs structures available, many questions remain regarding the actual mechanism of proline isomerization (6). In addition, little information is available about the surfaces involved in substrate binding in the regions outside the target prolines, and to date no structures are available for FKBPs bound to a native target peptide.

In the yeast Saccharomyces cerevisiae, the FKBPs are composed of four members (Fpr1–4) that differ in domain composition and cellular localization (10). Fpr1 is found in both the cytoplasm and nucleus. Similar to the prototypic FKBP12, Fpr1 encodes only a PPIase catalytic domain. The presence of a membrane-targeting signal N-terminal to the PPIase domain in Fpr2 results in compartmentalization to the endoplasmic reticulum (11).
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The translocation elongation factor analyses revealed a physical association of Fpr4 with ribosome repeat loci (11, 13). In keeping with a link to rDNA, interactome studies revealed an interaction of Fpr4 with several regions of rDNA and intergenic regions (New England Biolabs) from a modified pET-9d plasmid used for this often absent substrate element.

Fpr4(280–392) was expressed in S. cerevisiae—a common model system for studying nucleosome function, via a role as a histone chaperone (11, 12) as well as specific isomerization of proline residues in the N-terminal tails of histones (13). The N-terminal domain of Fpr4 enhances nucleosome assembly in vitro, which may explain its silencing effect on the rDNA loci in vivo (11, 12). The direct interaction of the Fpr4 N-terminal domain with both histone H3 and H4 N-terminal regions have been shown in vitro (12, 13) and thereby could explain the broad distribution of Fpr4 on constitutively active and induced genes (both promoter and transcribed regions) as well as on intergenic regions and rDNA repeat loci (11, 13). In keeping with a link to rDNA, interactome analyses revealed a physical association of Fpr4 with ribosome biogenesis factors (14, 15). The transcription elongation factor also associated with residues downstream from the target proline reveals a subdomain that differs from previous characterization of Fpr4 activity, and highlights a possible mechanistic importance for this often absent substrate element.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The catalytic C-terminal PPI domain from S. cerevisiae Fpr4 (residues 280–392) was cloned, expressed, and purified as previously described (17). In brief, Fpr4(280–392) was expressed in Escherichia coli BL21 LysY (New England Biolabs) from a modified pET-9d plasmid using LB or M9 minimal media supplemented with 15NH4Cl (1 g liter−1) and [13C6]glucose (2 g liter−1) with overnight expression at 22 or 30 °C, for LB or M9 cultures, respectively. Protein purification from cell lysate was achieved by using tobacco etch virus N-terminal Gly-Ala-Met-Ala- before Lys280. After dialysis into NMR buffer containing 20 mM sodium phosphate and 150 mM NaCl at pH 6.5.

Nitroanilide peptides were as described (13). Samples of residues downstream from the target proline revealed a substrate preference that differs from previous characterization of Fpr4 activity, and highlights a possible mechanistic importance for this often absent substrate element.

NMR Spectroscopy—Spectra were recorded at 292 K for structural studies and at 298 K for functional characterization, by using Bruker Avance III 700 MHz or 800 MHz NMR spectrometers, equipped with a triple resonance gradient standard probe or cryoprobe, respectively. Topspin 2.0 (Bruker BioSpin) was used for data collection and selected processing, with additional spectra processing by NMRPipe (18) followed by analysis with Sparky 3 (T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA) or NMRViewJ 8.0 (One Moon Scientific, Newark, NJ).

Structure Calculation—The backbone and side chain 1H, 13N, and 13C chemical shift assignments for Fpr4(280–392) were previously described (17). Spectra collected for distance restraints included a three-dimensional 13N-HSQC-NOESY and three-dimensional 13C-HSQC-NOESY with mixing times of 120 ms. Residual dipolar coupling data were measured by using interleaved spin-state selective TROSY, with anisotropy induced by 5% (w/v) C12E5 polyethylene glycol with an added 0.96 molar eq of 9-n-hexanol (19), resulting in a final water 1H–2H quadrupolar splitting of 35.5 Hz. The calculated residual dipolar coupling values were the average of three separate measurements. The ensemble of structures was calculated by using a standard ARIA 1.2/CNS 1.1 setup (20, 21). An initial calculation used a set of 2416 manually assigned NOESY peaks accompanied by 671 peaks with ambiguous assignment. Dihedral angles obtained from TALOS+22 were included when consistent with DANGLE predictions (23). Leucine residue χ angle restraints were included for side chains with 13Cβ and 13Cγ values indicative of limited conformational averaging (24).

Residual dipolar coupling values for 50 residues within predicted secondary structure elements and displaying [H3]13N-heteronuclear NOE values >0.6, were incorporated starting at iteration 4, and used values of −6.0 and 0.28 for the axial, Da, and rhombic, R, components, respectively, of the molecular alignment tensor. Hydrogen bonds were identified prior to the second round of structure calculation, and included only for amides displaying slow hydrogen-deuterium exchange, clearly present within α-helices or β-strands and consistent with NOE data. The 12 lowest energy structures following the final water refinement of 50 structures were taken as the calculated ensemble and have been deposited under code Protein Data Bank code 4BF8.

Histone H3 Peptides—Synthetic decapetides centered on three prolines from S. cerevisiae histone H3 were obtained from GeneCust (Dubelang, Luxembourg) and included acetylation and amidation of the N and C termini, respectively: ScH3(Pro−16) based on residues 11–20, Ac-TGGKAPRKQL-NH2; ScH3(Pro−30) based on residues 25–34, Ac-ARKASPPTGG-NH2; and ScH3(Pro−38) based on residues 33–42, Ac-GGVKKPHRYK-NH2. p-Nitroanilide peptides were as described (13). Samples were prepared from lyophilized peptide dissolved in 20 mM sodium phosphate and 150 mM NaCl at pH 6.5.

Peptide Chemical Shift Assignment—Peptide 1H chemical shift assignment on 2 mm samples and at 298 K used two-dimensional 1H,1H-TOCSY spectra in 10 and 99% 2H2O with a mixing time of 80 ms, as well as two-dimensional 1H,1H-ROESY spectra in 99% 2H2O with a mixing time of 200 ms. Aliphatic 1H and amide 15N chemical shift assignments were also aided by natural abundance two-dimensional 1H,15N-
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HSQC spectra in 99% 2H2O and 1H,15N-HSQC spectra in 10% 2H2O, respectively.

Rate Determination of Proline Isomerization—Samples of 2 mM peptides in NMR buffer containing 10% 2H2O were prepared in the presence of 10, 20, or 40 μM Fpr4(280–392). The NOESY spectra were recorded at 298 K at a 1H field strength of 700 MHz at different mixing times. The rate of proline isomerization was probed by measuring the intensities of the exchange cross-peaks correlating cis- and trans-specific proline 1H9 resonances. Based on the general relationship between the diagonal and exchange cross-peaks in the NOESY spectrum for a two-state transition (25) and assuming similar T1 values for the cis- and trans-proline peptides, the ratio between the exchange cross-peak (IEX) and the trans-conformer diagonal peak (ITRANS) can be described by the equation,

\[ \frac{I_{EX}}{I_{TRANS}} = \frac{1 - e^{-k_{ex}}} {1 - \frac{x_{cis}}{x_{cis}} + e^{-k_{ex}}} \]  

(Eq. 1)

kEX is the chemical exchange rate defined as the sum of back and forward rates of the two-state model, τmix is the mixing time, and xcis is the fraction of cis-conformer. The raw data were fit against Equation 1 using the least squares method (Levenberg-Marquardt algorithm provided by Igor Pro version 6.03) with \( k_{ex} \) as the free fit parameter and xcis based on observed values. To determine the cis and trans proline populations one-dimensional 1H spectra were measured at 298 K with subsequent signal integration using Topspin 2.0 on at least two independent spectra.

Affinity Measurement—Titration of 150 μM [15N]Fpr4(280–392) in NMR buffer with proline or histone H3 peptides used stock solutions of 150 mM substrate in NMR buffer, added at final concentrations of 0, 0.25, 0.5, 1, 2.5, 5, 7.5, and 10 mM (except for ScH3[Pro-160]). Proline was additionally titrated with 15 and 20 mM. For each titration point a 1H,15N-HSQC spectrum at 298 K was collected. Calculation of Kd values used the standard equation of fast exchange binding (26) for Equation 2,

\[ \Delta \delta = \frac{A(L + P + K_d) - \sqrt{(L + P + K_d)^2 - 4LP}}{2P} \]  

(Eq. 2)

where L and P are the ligand and protein concentrations, respectively, and Δδ is the combined 1H9 and 15N amide chemical shift perturbation in Hz, where Δδ = (ΔδH + ΔδN)1/2. The affinity, Kd, and the perturbation of the fully bound complex, A, are obtained by a non-linear fit by using the Levenberg-Marquardt algorithm within QtIplot version 0.9.7.10. Maximum chemical shift values obtained from the fit for Trp345/Ile342 are 250 ± 100/160 ± 50, 250 ± 20 ± 180 ± 10, and 90 ± 10/60 ± 20 Hz for proline, ScH3[Pro-160], ScH3[Pro-30], or ScH3[Pro-38], respectively.

Paramagnetic Relaxation Enhancement—Two peptides based on ScH3[Pro-30] were designed in which either the first or last residue was mutated to cysteine (A25C or G34C, respectively). 100 μl of 10 mM 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidineoxyl radical (iodoacetamido-PROXYL; Sigma) in methanol was added to an equal volume of 10 mM peptide dissolved in 10 mM Tris (pH 8.0). After 1 h at room temperature, the peptide was dried by vacuum centrifugation at 30°C for 2 h. The modified peptides were each dissolved in NMR buffer (20 mM sodium phosphate, pH 6.5, 150 mM NaCl). The final samples of 500 μl contained 100 μM [15N]Fpr4(280–392), 2 mM spin-labeled peptide, and 10% 2H2O added for the lock. After collecting 1H,15N-HSQC spectra of the oxidized form, 25 μl of 100 mM ascorbic acid in NMR buffer (adjusted to pH 6.5) was added to make a final concentration of 5 mM. Following a 1-h incubation, reduced form 1H,15N-HSQC spectra were collected. The experiments were performed in duplicate, and the average derived paramagnetic relaxation enhancement values are based on the intensity ratios of the reduced to oxidized forms adjusted by the small dilution factor following ascorbic acid addition.

RESULTS

Solution Structure of the Fpr4 PPlase Domain—Previous studies of Fpr4 have shown that the isolated C-terminal region from residue 280 to the final residue 392 (hereafter named Fpr4(280–392)) can be recombinantly produced in bacteria and comprises an independently folded domain (17). This construct retains the ability of the full-length protein to inhibit in vitro Set2 methylation of a histone H3 peptide (13). To further characterize the proline isomerization activity specific to the C-terminal region, we first calculated an ensemble of 12 well defined structures of Fpr4(280–392) from a combination of NMR spectroscopy data including distance, dihedral, and orientational restraints (Fig. 1A, Table 1).

The FKBP family of PPlases share a canonical domain fold consisting of a β-sheet composed of four large and two small β-strands, opposed by a single main α-helix (27, 28). This architecture is also present in Fpr4(280–392) (Fig. 1B). An additional but less well defined N-terminal strand, β0, lies adjacent to strand β1 to further extend the β-sheet for the Fpr4 PPlase domain. A small helical region is also present between β5 and β6. Structural alignment reveals a very high similarity to other members of the FKBP family represented in the PDB including a 0.7-Å root mean square deviation to the prototypic FKBP12 structures (PDB code 2PPN), as well as a 0.9-Å root mean square deviation to the more distant FKPA from E. coli (PDB code 1Q6U), even though there is only a modest 30% overall sequence similarity (Fig. 1C).

A notable feature of the FKBP catalytic domain is a prominent hydrophobic pocket that is thought to bind the proline, an hypothesis based mainly on structures with the bound inhibitors FK506 and rapamycin (6, 29–32). A recent structure of the Plasmodium vivax FKBP35 in complex with a proline-containing substrate provides a confirmation of this assumption (33). Of the 32 residues identical in the PPlase domains from Fpr4, human FKBPI2, and E. coli FKPA, 10 residues surround this hydrophobic pocket and have similar side chain placements in the three displayed structures (Fig. 1D). Specifically, Trp345 lies at the bottom of the hydrophobic pocket, with the additional residues Tyr315, Phe325, Phe332, Phe334, Val341, Ile342, Tyr368, and Phe384 forming the sides of the proline-binding cavity. The final residue, Asp324, provides a contrast to the largely hydrophobic nature of the cavity but nevertheless is a conserved
residue and common feature of the PPIase domain, and thus may serve a role in the catalytic mechanism. Asp324 along with the less-conserved Glu340 together comprise a small acidic region at the side of the hydrophobic pocket in Fpr4 that is otherwise surrounded by a large surface region enriched in positively charged amino acids (Fig. 1E).

Activity toward Histone Proline Residues—The N-terminal region of histone H3 has already been shown to contain substrate proline residues for Fpr4 (13). To confirm the activity of Fpr4(280–392) by using NMR spectroscopy, we have designed suitable decapeptides centered around the peptidyl-prolyl bond of the three prolines from the N-terminal tail of histone H3 from S. cerevisiae (Fig. 2A): Pro16 (ScH3(Pro-16)), Pro30 (ScH3(Pro-30)), and Pro38 (ScH3(Pro-38)). The inclusion of five residues to each side of the substrate peptidyl-prolyl bond ensures that natural contacts between the PPIase domain and proline-flanking features will be preserved.

As a first step to activity measurement, the complete 1H, 13C, and 15N assignments were obtained for all peptides using standard NMR spectroscopy (data not shown). In each case, the clear observation of resonances from both the trans and cis conformations of the central proline enabled quantification of the cis-Pro populations. Under these conditions each peptide has an equilibrium cis-Pro population of ~6% (Fig. 2B) in keeping with general values reported in literature (34–38).

In addition to measurement of the conformation populations, NMR spectroscopy is particularly applicable to obtain values for the rate of cis-trans isomerization for the peptidyl-prolyl bond. Specifically, exchange between two populations (i.e., the peptides containing trans-Pro or cis-Pro) can be quantified by using two-dimensional ROESY or NOESY experiments. In these spectra, the size of the exchange cross-peaks connecting the two states of the same nuclei represent the amount of isomerization that occurs during the experimental observation times. The two hydrogen nuclei on the δ carbon (Fig. 2C) are especially useful in this regard due to the large difference in chemical environment for these nuclei in the trans and cis conformations. For the isolated peptides at ambient

FIGURE 1. Solution structure of Fpr4(280–392). A, ensemble of 12 low energy structures for Fpr4(280–392). B, lowest energy structure with labeled secondary structure elements. C, sequence alignment of S. cerevisiae Fpr4 with human FKBP12 (UniProt number P62942) and E. coli FKPA (UniProt number P45523). Residues highlighted in black are conserved in all three species. Asterisks indicate the natural N and C termini of the protein. The secondary structure of Fpr4(280–392) is displayed above the alignment. Residues that line the hydrophobic pocket are indicated with open circles. D, close-up view of the hydrophobic pocket with selected residues of Fpr4(280–392) (red) superimposed with those from human FKBP12 (PDB code 2PPN; green) and E. coli FKPA (PDB code 1Q6U; violet). These residues correspond to the open circles in C and are annotated with the amino acid type and sequence number. At the bottom of the panel, the location of the hydrophobic pocket is indicated on the charge surface representation of Fpr4(280–392), with acidic regions in red and basic regions in blue, and the orientation shown on the left equivalent to that displayed in A and B.
Temperature, this isomerization is usually on the order of minutes to hours (39, 40). As expected, there is indeed no substantial isomerization observed for the three peptides during periods up to seconds as evident by the lack of the exchange cross-peak (Fig. 2D).

Upon addition of catalytic amounts (10 μM) of Fpr4(280–392) the isomerization rate is significantly increased. Evidence of the cis–trans exchange is now observed for all three peptides (Fig. 2E, H1, and I). In keeping with a mechanism by which Fpr4 acts by reducing the activation energy of isomerization, this increased conversion rate does not alter the equilibrium populations of cis-Pro for the three peptides, which remain at 6% (Fig. 2B). To confirm that the cross-peak is indeed due to chemical exchange, a ROESY spectrum was also collected and in keeping with chemical exchange the sign of the cross-peak is the same as the diagonal peaks (Fig. 2F). In contrast, cross-peaks between neighboring hydrogens display an inverted sign. The catalytic increase in proline isomerization is dependent on the activity of the added Fpr4(280–392) because the addition of 1.5 molar eq of the FKBP inhibitor rapamycin eliminated the observed cis–trans conversion (Fig. 2G).

**Equilibrium Rate Determination by NMR**—To better understand the molecular determinants of the interaction between Fpr4 and peptide substrates, we expanded and quantified the results from the NMR-based analysis of cis–trans isomerization (Fig. 2). By using variable amounts of Fpr4(280–392) from 10 to 40 μM, and using five mixing times from 0 to 400 ms, we fit the resulting data to curves representative of an exchange between two states (Fig. 3A and “Experimental Procedures”). From the fit for each sample condition, a combined rate, \( k_{\text{EX}} \), was derived, which is the sum of the individual trans–cis and cis–trans isomerization rates. For each peptide, the slope of these derived rates as a function of added Fpr4(280–392) was used to calculate the specific catalytic activities toward the three substrate peptides (Fig. 3B).

Two of the peptides, \( \text{ScH}^3(\text{Pro}^{16}) \) and \( \text{ScH}^3(\text{Pro}^{30}) \) display similar enhancement of isomerization rate upon addition of Fpr4(280–392), with \( k_{\text{EX}} \) values of 233,000 ± 14,000 M\(^{-1}\) s\(^{-1}\) and 236,000 ± 24,000 M\(^{-1}\) s\(^{-1}\), respectively. In contrast, the third peptide, \( \text{ScH}^3(\text{Pro}^{30}) \), was a significantly poorer substrate for Fpr4(280–392), with a rate constant of 72,000 ± 29,000 M\(^{-1}\) s\(^{-1}\). For Pro\(^{30}\) and Pro\(^{38}\), these results are generally comparable with a previous characterization of Fpr4 and histone H3 substrates, however, there is a noted discrepancy for Pro\(^{16}\), for which the isomerization rate in this previous study was unaffected by the addition of Fpr4 (13). It is significant, however, that in this previous study the rate analysis was performed by using a spectrophotometric assay based on quantitative cleavage of substrates with a C-terminal X-Pro-Phe-pNA in which only the trans-Pro peptide conformation is recognized by chymotrypsin (41). An inherent limitation of this method is the requisite truncation of the substrate peptides, such that the native sequence C-terminal to the proline is absent. In addition, NMR spectroscopy allows for observation of the equilibrium values of the combined cis–trans and trans–cis transitions \( k_{\text{EX}} \) instead of the cis–trans specific rate constant \( k_{\text{cat}}/K_m \) in the protease-coupled assay.

To distinguish between differences in the nature of the substrate instead of the analytical technique that was used, we subjected the substrates of the chymotrypsin-based assay to the same NMR spectroscopy approach as described for the decapeptides. Each of the p-nitroanilide molecules indeed show enhanced proline isomerization as a result of added Fpr4(280–392) (Fig. 4). Based on the intensity of the exchange cross-peak, the NOESY spectra indicate the highest efficiency toward Pro\(^{30}\) (Fig. 4B). Using the protocol described for the three decapeptides, we quantified the isomerization and found that addition of Fpr4(280–392) resulted in a rate constant of 68,000 ± 7,000 M\(^{-1}\) s\(^{-1}\) for \( \text{ScH}^3(\text{Pro}^{30}) \)-pNA. Similar to the chymotrypsin-based analysis of the pNA peptides (13), the molecules based on Pro\(^{16}\) and Pro\(^{38}\) were poorer substrates (Fig. 4, A and C). For \( \text{ScH}^3(\text{Pro}^{30}) \)-pNA the \( k_{\text{EX}} \) value is only 13,000 ± 3,000 M\(^{-1}\) s\(^{-1}\). The value for \( \text{ScH}^3(\text{Pro}^{16}) \)-pNA is also of the same order but cannot be reliably fit to the data.

There are two important conclusions to be made from the analysis of the decapptide versus p-nitroanilide substrates. The first finding is that the same trend is observed for the three pNA substrates whether the analytical technique is based on NMR spectroscopy or the chymotrypsin assay. As a result, the discrepancy between the decapptides and pNA peptides can be attributed to the design of the substrates and likely the presence or absence of residues C-terminal to the proline. The second observation is that the highest \( k_{\text{EX}} \) value determined for the pNA substrates (68,000 ± 7,000 M\(^{-1}\) s\(^{-1}\) for \( \text{ScH}^3(\text{Pro}^{30}) \)-pNA) is only at the level of the least efficient decapptide (72,000 ± 29,000 M\(^{-1}\) s\(^{-1}\) for \( \text{ScH}^3(\text{Pro}^{30}) \)). In addition, the equilibrium...
cis-Pro populations for the three peptides (23% for ScH3(Pro-30)-pNA and 15% for ScH3(Pro-16)-pNA and ScH3(Pro-38)-pNA) are significantly higher than the 6% observed for all three decapeptides. These findings together highlight atypical aspects of these particular substrates, and advocate the use of native peptide sequences flanking both sides of the central proline to better represent the native interaction between Fpr4 and histone H3. The results also prompted us to further probe the nature of the substrate peptides at the molecular level.

**Substrate Conformational Requirements**—We next compared the structural features of each histone tail decapeptide. To this end, two-dimensional ROESY NMR spectroscopy, which measures local 1H contacts, was performed. In this experiment, spectra cross-peaks of the opposite sign as the diagonal are primarily observed for nuclei that are consistently within a short distance (less than 6 Å). Atoms with substantial local dynamics or larger conformational flexibility are therefore unlikely to give rise to clear 1H–1H contacts. In the three peptides, there are a number of expected cross-peaks for 1H within the same side chain (Fig. 5) because these distances are constrained by covalent bonds. In contrast, there are striking differences in the number of 1H contacts observed for sequential or most distant residues in the three peptides (Fig. 5).

The reduced flexibility of the central peptidyl-prolyl bond is reflected by the consistent observation of contacts between the proline Hδ hydrogens and the hydrogen nuclei belonging to the previous residue. Some additional but weak cross-peaks were also observed for two other sequential residues in ScH3(Pro-16), but otherwise both ScH3(Pro-16) and ScH3(Pro-30) are devoid of long-range order. Therefore these regions of the histone H3 peptides are conformationally disordered in solution under these conditions. In contrast, ScH3(Pro-38) is considerably less flexible: it displays a significant number of observable 1H–1H through-space contacts for sequential as well as i+2 residues surrounding Pro38 and extending to the C-terminal region. The rigidity in ScH3(Pro-38), particularly in the region C-terminal of the proline, may explain why this peptide is least labile to Fpr4 isomerization. Notably, because this region is deleted in the spectrophotometric assay substrates this may reconcile why Fpr4 was previously reported to target Pro38 with preference over Pro16 (13). Taken together these results demonstrate that in solution the Pro38 region is more structurally constrained than regions about Pro16 and Pro30. Furthermore, recognition of substrate prolines in these regions may be influenced by such conformational constraints.

**Substrate Affinity**—NMR spectroscopy can provide quantitative determination of weak binding affinities, such as those that mediate encounters between enzymes and substrates. We therefore decided to also measure the affinity of Fpr4(280–392)
for each histone peptide. Titration of $^{15}$N-labeled protein by the three histone H3 decapeptides, as well as isolated proline, was followed by recording sequential $^1$H,$^{15}$N-HSQC spectra (42). Comparison of the spectra (Fig. 6A) revealed only minor global changes in the cross-peak positions with the greatest perturbation observed for the Trp345 side chain and the neighboring Ile342 backbone amide. The position of these atoms (Fig. 1D) at the bottom and side of the hydrophobic pocket, respectively, are in keeping with a possible close proximity to the bound substrate. A fit of the chemical shift perturbation for Trp345 and Ile342, as a function of substrate concentration, is based on the equation for fast exchange binding (Fig. 6B and “Experimental Procedures”). The highest affinities are observed for $\text{ScH}_3(\text{Pro-30})$ and $\text{ScH}_3(\text{Pro-38})$ with values of 25 and 29 mM, respectively, although due to limited solubility of Fpr4(280–392) we were unable to reach reliable estimates of the fully bound chemical shifts thus affecting the quality of fit. Nevertheless, the remaining peptide $\text{ScH}_3(\text{Pro-16})$ displays a significantly lower affinity that is estimated at 90 mM. For comparison, the affinity of Fpr4(280–392) for simple proline is also relatively weak (110 mM). It is of interest that the order of histone peptides from highest to lowest affinity ($\text{ScH}_3(\text{Pro-30}) > \text{ScH}_3(\text{Pro-38}) > \text{ScH}_3(\text{Pro-16})$) does not follow the order of substrate activity ($\text{ScH}_3(\text{Pro-16}) > \text{ScH}_3(\text{Pro-30})$).
It should also be noted that the observed affinity represents a combined measurement for peptides containing both the cis and trans proline conformations. A previous comprehensive study on the parvulin Pin1 isomerization of an amyloid-based ligand quantified a higher affinity for the cis-Pro containing peptide (43). This finding led to a putative mechanism by which the higher affinity for the less populated conformation would thereby allow balanced access to the isomerase by both conformations. To precisely calculate the conformation-specific binding affinities it would be necessary to add increasing amounts of Fpr4(280–392) to the peptide and follow the chemical shift of the cis- and trans-specific peaks (such as the δ-hydrogens of the proline as used for the isomerization rate analysis). However, the limited solubility of Fpr4(280–392), with a maximum around 200 μM, precludes this type of analysis.

**Molecular Details of Substrate Binding**—To supplement the binding information obtained from the analysis of affinities, we decided to obtain molecular details of the association surface between Fpr4(280–392) and the substrate peptides. During our titration studies by using NMR spectroscopy, it was found that the largest chemical shift perturbation was attributed to the indole side chain of Trp345 (Fig. 6) and also to the backbone amide of Ile342. The tryptophan lies at the bottom of the hydrophobic cavity that is presumed to interact with the proline (Fig. 1D), and in particular the H41-N41 atoms are positioned in the center of this cavity and would be expected to be in the closest proximity to any bound substrate. The backbone amide of Ile342 is located along the side of the hydrophobic cavity, which would also place it close to the bound substrate. Moreover, the recent crystal structure of *P. vivax* FKBP35 bound to succinyl-ALA-Leu-Pro-Phe-p-nitroanilide (sALPF-pNA) shows that the equivalent backbone amide (Ile274 in *Pv*FKBP35) forms a hydrogen bond with the substrate proline carbonyl oxygen. For the remainder of the protein during the various titrations there are broad regions of small chemical shift perturbation, including around His302, which is highly sensitive to unrelated minor changes in pH and unlikely to report on actual binding events. To better determine the region of substrate binding on Fpr4(280–392) we decided to use a more direct method to probe association between the PPI domain and the substrate.

The presence of a paramagnetic compound (such as a nitroxide moiety) in a biomolecule or complex causes increased relaxation during NMR spectroscopy and thereby reduced cross-peak intensity for atoms within 15–20 Å in a distance-dependent manner. As a result of this property, the technique can be used to determine longer range distances even in samples where a high level of dynamics or a low affinity is observed (44). To use this approach to study the interaction between peptide substrates and Fpr4(280–392), we designed two peptides based on Sch3-Pro30 in which either the first or last residue was mutated to a cysteine (Fig. 7A). This peptide was chosen because it combined both high affinity and was an efficient substrate. A nitroxide compound was covalently attached to the cysteine side chains and the paramagnetic relaxation enhancement (PRE) observed for amides within [15N]Fpr4(280–392). For each ligand there were several locations of significant PRE, however, when the two data sets were compared there were regions that have PRE effects specific to the spin label attached to the N-terminal or C-terminal residue of the peptide (Fig. 7A). From these data, the relative locations of the substrate N and C termini can be mapped to the PPIase domain surface (Fig. 7B).

The derived contact regions show similarity to the structural details of the sALPF-pNA ligand bound to *P. vivax* FKBP35 (Fig. 7C) (33). In both cases, the N-terminal region extends to one side of the hydrophobic pocket in a surface region that is also occupied by inhibitor binding such as with rapamycin to human FKBP12 (Fig. 7D) (29). The C-terminal interaction region appears to be primarily located on the opposing side of the proline-binding pocket. However, it should also be noted that only the cis-Pro conformer details are described in the *Pv*FKBP35 complex, and therefore it is not known how the trans form is recognized by the PPIase domain. Indeed this conformational heterogeneity of the substrate will likely cause multiple regions of PRE on the surface of Fpr4(280–392) that cannot be unambiguously assigned to a particular proline conformation.

**DISCUSSION**

The characterization of the isolated PPI domain from *S. cerevisiae* Fpr4 reveals a canonical fold and activity toward three peptide substrates derived from the N-terminal region of histone H3. We found that measurements of binding affinity contrasted with those of catalytic efficiency, providing different rankings of the three proline-centered substrates. Focusing on the central prolines, Pro30 displays the highest affinity, followed by Pro16 then Pro38. In terms of catalytic efficiency, Pro16 and Pro30 have equally high increases in isomerization rates, whereas Pro38 shows little response to the addition of Fpr4. In addition, the ranking of substrate preference for Fpr4 using the pNA peptides was more similar to the binding affinity results.

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**FIGURE 6. Substrate affinity to Fpr4(280–392).** A, overlay of the Trp345 side chain amide peak (H41-15N) from the series of 1H, 15N-HSQC spectra used to follow the titration of 150 μM 15N-labeled Fpr4(280–392) with increasing concentrations of proline, Sch3-Pro30, or Sch3-Pro38. B, determination of binding affinity by using the combined 1H and 15N chemical shift perturbation in Hz of the Trp345 side chain amide peak from A as a function of ligand concentration. Curve-fitting used the equation described under “Experimental Procedures” to derive values of $K_d$ and maximum chemical shift perturbation.
These various findings prompt questions into the nature of these differences, including the aspects of the catalytic mechanism that may be involved, as well as the extent to which these differences are specific to Fpr4 or more generally applicable to the FKBP family.

The measurement of binding affinity for proline isomerases such as Fpr4 is complicated by several aspects. For the isolated substrate, the two proline conformers exist in equilibrium and presumably bind to the PPIase domain with different affinities. A second equilibrium also exists for the bound substrate, so that proline conformers are exchanged during contact with Fpr4. As a consequence, some of the substrate molecules will bind and dissociate while in the same conformation, whereas other molecules will bind in one conformation and dissociate in the alternate conformation due to isomerization while in complex with Fpr4. Although it has been possible to determine the conformer-specific affinities in the interaction between Pin1 and a phosphopeptide from the amyloid precursor protein (43), similar analyses with FKBP have not been reported. In the study of Fpr4(280–392) we were unable to determine conformer specific affinities mainly due to solubility limits of the PPIase domain.

In the current study, we have determined the overall apparent affinity of the peptides by using NMR spectroscopy and chemical shift perturbation. These $K_d$ values are the combination of both the cis- and trans-specific affinities. For Pin1, the affinity toward the cis-conformation is higher than that of the trans-Pro peptide (43). If this condition holds true for Fpr4, then we can place an upper limit on the cis-conformer affinity by fitting the data from Fig. 5 solely to the concentrations of the tighter binding cis-Pro substrate (i.e. 6% of the total peptide concentration). This results in maximum cis-specific affinities of 1.2 ± 0.1, 1.5 ± 0.5, and 4 ± 1 mm for ScH3(Pro-30), ScH3(Pro-38), and ScH3(Pro-16), respectively. Even using these upper limits, it is clear that there is only weak binding between the PPIase domain and peptide substrates.

If the main function of the FKBP domain toward histone H3 is catalysis of proline isomerization, then it is possible that this low affinity is sufficient for this enzymatic role. However, increased specificity likely requires auxiliary domains to direct Fpr4 to a more restricted substrate protein repertoire. For example, the differential interaction of mammalian FKBP51 and FKBP52 with steroid hormone receptor complexes is mediated by their tetratricopeptide repeat domains and C-terminal divergent sequences (45). As another example, the insert-in-flap (IF) domain (46) from *E. coli* SlyD confers chaperone activity to this FKBP enzyme and transfer of this domain to the human FKBP12 is sufficient for improved substrate affinity by the chimeric protein (47). Similar effects have been noted for the transfer of chaperone domains from non-FKBP proteins.

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**FIGURE 7. Interaction details between PPI domain and peptide substrates.** A, location of the nitroxide spin labels on the ScH3(Pro-30)-based peptides. The structure shows all amide hydrogen atoms in Fpr4(280–392) as spheres, with colors based on paramagnetic relaxation that is specific to the N-terminal or C-terminal placement of the nitroxide spin label. B, charge surface representation of Fpr4(280–392), where red regions are acidic and blue regions are basic. C, structure of the *P. vivax* PPI domain in complex with a succinyl-Ala-Leu-Pro-Phe-p-nitroanilide substrate with a cis-proline conformation (PDB code 4ITZ (33)). The location of the N and C termini of the modified peptide is indicated. D, location of inhibitor binding illustrated by using the complex of human FKBP12 with FKS06 (PDB code 1FKF (29)).
such as GroEL and SurA (48). For Fpr4, the N-terminal domain may function in a similar capacity to restrict the substrate repertoire. However, this region may also serve a separate and distinct role in the full-length protein, because the N-terminal domain displays a isolated histone chaperone activity (11, 12). This property of the N-terminal region has also been observed for the orthologous proteins FKBP39 from Schizosaccharomyces pombe (11) and FKBP53 from Arabidopsis thaliana (49).

Apart from a catalytic role, it is also possible that the main function of the PPI domain of Fpr4 may be in simple binding of histone H3 via proline recognition. From the estimation of yeast protein abundance, S. cerevisiae contains ~14,000 molecules of Fpr4 per cell (50), which corresponds to a concentration of roughly 8 μM if present mainly in the nucleus (estimated nuclear volume of 2.9 μm³ based on Ref. 51). With millimolar affinities toward the histone H3 peptides, it is therefore unlikely that the PPlase domain alone will substantially occupy binding sites on target sequences. One possibility is that the presence of the N-terminal region provides additional affinity to the histone such that the combined interaction of the N-terminal and PPlase domains provides sufficiently tight binding. It is also possible that the binding affinity may be increased via incorporation with multiprotein complexes harboring these auxiliary association domains.

The quantity of Fpr4 in the cell is also similar to the estimated concentration of another histone chaperone, Nap1 (~8,000 molecules (50)). Because there are around 70,000 nucleosomes per yeast cell (52), each of these chaperones are therefore present at levels of 10 to 20% with respect to nucleosome molecules. Such amounts would be in keeping with a role as a histone chaperone in nucleosome regulation. However, the interactome studies have identified various binding partners, and it is possible that Fpr4 instead plays its main role within larger biomolecular complexes. For example a large proportion of Fpr4 may be sequestered in ribosomal protein complexes, leaving only a smaller pool to engage in chromatin regulation.

The fact that the three histone H3 peptides have different rankings based on affinity or catalytic efficiency also has implications with respect to Fpr4 function. It may be that in certain cases an isomerase role is required, whereas in other cases aspects of affinity have increased importance. Such a scenario may be involved in the interplay between Fpr4 and the histone methyltransferase Set2. Through site-directed mutation, it was found that the presence of Pro38 was necessary to retain Fpr4-methyltransferase Set2. Through site-directed mutation, it was found that the presence of Pro38 was necessary to retain Fpr4-

models. In our investigation it was found that residues C-terminal to the proline may affect the efficiency of the Fpr4 PPlase domain to enhance the rate of isomerization. The structure of sALPF-pNA bound to P. vivax FKBP35 (33) presents the molecular details of a peptide-based substrate with the proline in a cis-conformation. In the trans position it is not known which changes would occur in the contacts between the PPlase domain and the sALPF-pNA. One possible mechanism dictates that a part of the substrate peptide remains in contact with the PPlase domain in both conformations, whereas the other terminus experiences mobility necessary to accommodate the 180° change in the peptide backbone. The importance of the residues C-terminal to the proline, and their disordered nature in the Sch3(Pro10) and Sch3(Pro30) peptides, may indicate that their mobility is a required aspect. As such, the C terminus may experience dramatic changes between the cis-Pro and trans-Pro containing substrates. Therefore the N-terminal residues would be the anchor part of the substrate peptide, which may explain why the binding results match well with the findings based on the p-nitroanilide substrates.

Continued investigation into the catalytic mechanism of Fpr4 will help to elucidate the contribution of the residues both N- and C-terminal to the central proline. In addition, using native peptides in the study of other FKBP enzymes will also help reveal commonalities to the isomerization mechanism. At the same time, details that are unique to Fpr4 will be separated from aspects that are applicable to the general FKBP family.

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*Structure and Activity of Fpr4 Catalytic Domain*