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Crystallographic Proof for an Extended Hydrogen-Bonding Network in Small Prolyl Isomerases

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Supporting Information

ABSTRACT: Parvulins compose a family of small peptidyl-prolyl isomerases (PPIases) involved in protein folding and protein quality control. A number of amino acids in the catalytic cavity are highly conserved, but their precise role within the catalytic mechanism is unknown. The 0.8 Å crystal structure of the prolyl isomerase domain of parvulin Par14 shows the electron density of hydrogen atoms between the D74, H42, H123, and T118 side chains. This threonine residue has previously not been associated with catalysis, but a corresponding T152A mutant of Pin1 shows a dramatic reduction of catalytic activity without compromising protein stability. The observed catalytic tetrad is strikingly conserved in Pin1- and parvulin-type proteins and hence constitutes a common feature of small peptidyl prolyl isomerases.

Parvulins are small peptidyl-prolyl cis/trans isomerases (PPIases) conserved from bacteria to humans. They are involved in cellular processes such as cell-cycle regulation and cancer, protein quality assessment and turnover as well as protein-folding disorders such as Parkinson's and Alzheimer's diseases,[1,2] and also in maintaining the pluripotency of stem cells.[3] In terms of substrate specificity, phosphospecific (Pin1-type) and non-phosphospecific (*Escherichia coli*-type/Par14-type) parvulins are distinguished.[4,5]

Structurally, all parvulins known to date show a typical $\beta\alpha\alpha$h$\beta\alpha\beta\beta$ folding topology and share a number of highly conserved amino acids within their catalytic centers.[6−11] For Pin1, cysteine C113 was originally postulated to bind covalently to the substrate Xaa−Pro bond during isomerization.[6] Nonetheless, a cysteine at the respective position of human Pin1 seems not to be required for function in the orthologous protein Ess1 in yeast.[12] Moreover, the respective C70 in the *Arabidopsis* Pin clearly points away from the active site.[8] Finally, this cysteine residue is replaced by an aspartate at the corresponding position in several other parvulins.[5,7,11]

In addition, there are two conserved histidines within the catalytic cleft of Pin1. Together with C113, the side chain of H59 is in close proximity to a bound Ala−Pro dipeptide,[6] and the respective H59A mutant displays a remarkable reduction in activity.[13] If these residues are necessary to stabilize a tetrahedral intermediate,[6] deprotonation of H59 and protonation of H157 would be required. Indeed, a strong pH dependence of the activity was observed for Pin1.[6]

Contrary to the above, the two histidines were recently reported merely to stabilize the overall fold of Pin1.[14] While an H59L mutant of human Pin1 failed to compensate for the loss of Ess1 function in yeast, the double mutant H59L/H157L did. These genetic effects were attributed to significantly reduced protein stability of the H59L mutant and partial rescue of the stability by the double mutant.[14] Despite a considerable amount of biochemical and structural work, key elements of the catalytic mechanism of parvulins remain unresolved.

Presenting a crystal structure of the Par14 PPIase domain at 0.8 Å resolution, we now analyze side-chain conformers and the full hydrogen-bonding network within the catalytic cavity of small prolyl isomerases. Our structural data identify the backing T118 as a previously unrecognized building block of the active site. The importance of this supporting residue was subsequently tested with corresponding point mutants of the mitotic regulator Pin1, as a sensitive assay is available for this enzyme but not for Par14.

Crystals of the catalytic domain of human Par14 diffracted very strongly, and the final structural model was refined to a resolution of 0.8 Å (Supplementary Table 1). Gel filtration and crystal contact analysis with PISA[15] revealed that the crystallized Par14 catalytic domain is monomeric. The crystal structure invigorates the previous NMR-based finding that the peptide bond between D113 and P114 is in the cis conformation,[7] resolving an ambiguity with respect to another published NMR structure of Par14.[16] The active site is mainly hydrophobic in character (residues L44, L82, M85, M90, V91, F94, and F120), with the exception of a negatively charged patch around D74 and the polar residues H42, T118, and H123 (Figure 1A) that are part of the active site. The observed conformation of the side chains leads to an enlarged substrate binding pocket similar to the one observed in the PPIase domain of PrsA from *Bacillus subtilis*.[17]

As the diffraction data extended to subatomic resolution, it was possible to discern the positions of hydrogen atoms in many places. Electron density can clearly be seen for the hydrogen atoms of H42 and H123 in the active center (Figure 1B), undoubtedly defining the flipping state of the imidazole rings, their tautomeric state, and the existing hydrogen-bonding pattern. The side chains of residues H42, D74, T118, and H123 interact through the hydrogen-bonding network D74-O···H−N_{E2}−H42−N_{D1}···H−N_{D1}−H123−N_{E2}···HO-T118 (Figure 1B). The hydroxyl

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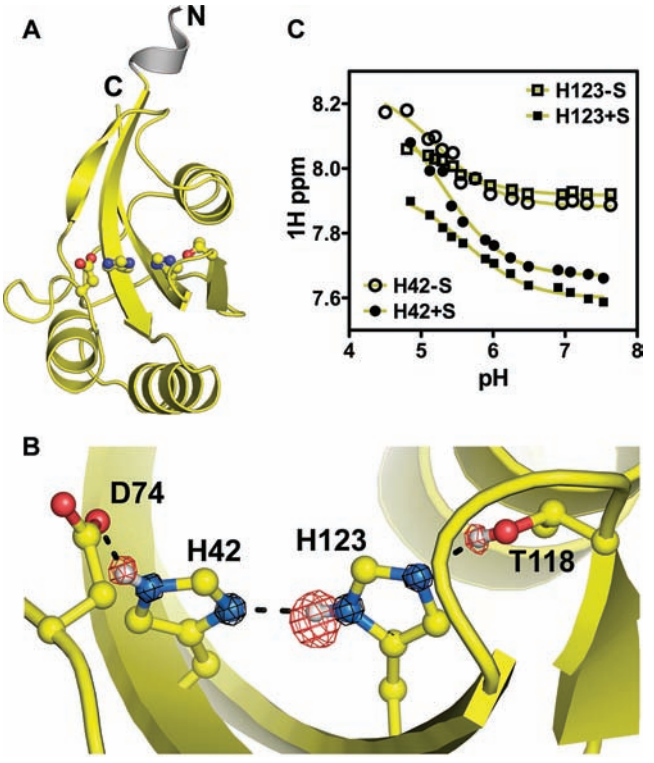

Figure 1. The subatomic-resolution structure of the PPIase domain of Par14 reveals a tetrad of hydrogen-bridged residues. (A) The Par14 crystal structure shows the $\beta\alpha\alpha\mathrm{h}\beta\alpha\beta\beta$ fold typical of parvulins. All amino acids including the cloning linker[20] (depicted in gray) could be traced completely in the electron density. The polar residues D74, H42, H123, and T118 within the substrate binding pocket are shown as ball and stick models. (B) The H-bridged tetrad within Par14. The hydrogen-bonding pattern from D74 via H42 and H123 to T118 is clearly visible. The black density is from an $|F_o - F_c|$ map (displayed at 11σ) of a model from which the side-chain atoms of H42 and H123 were removed and the coordinates perturbed prior to refinement to determine the orientation of H42 and H123 by locating the nitrogen atoms. The red density represents an $|F_o - F_c|$ map (displayed at 3σ) generated in a similar manner to demonstrate the observation of hydrogen atoms in the high resolution data set. (C) ^{1}H chemical shifts for the H_{E1} protons of the imidazole moiety plotted vs pH for histidines H42 and H123 in Par14 with and without Suc-ARPF-pNA (S). The corresponding titration of Pin1 with Ac-AApSPR-pNA is shown in Supplementary Figure 1. The derived microscopic pK_a values for the histidine residues in Par14 and Pin1 in the presence and absence of the model substrates are given in Table 1. (A) and (B) were prepared using PyMOL version 1.3 (Schrödinger, LLC).

group of T118 is supported by hydrogen-bonding to the main-chain amide of F120. The two histidine residues H42 and H123 of Par14 correspond to the above-mentioned H59 and H157 in Pin1, respectively.

Hydrogen-bonding networks as observed in our Par14 structure often result in shifted pK_a values. Therefore, we determined the microscopic pK_a values of the conserved histidine side chains in Par14 and Pin1 from pH titrations monitoring the H_{E1} shift of the imidazole groups by NMR spectroscopy (Figure 1C and Supplementary Figure 1). Overall, the pK_a values for the two core histidines in Par14 are nearly one pH unit lower than those in Pin1 (Table 1). Most likely, these reduced intrinsic pK_a values are due to formation of a hydrogen bond with the acidic D74, which is replaced by C113 in Pin1.

Table 1. Titration of Histidines in Parvulins and Bimolecular Rate Constants of Pin1 Mutants[a]

Microscopic pK_a Values of Histidine Imidazole Groups			
parvulin	histidine	pK_a within free enzyme	pK_a with bound substrate[b]
Par14	H42	5.17 ± 0.13	5.27 ± 0.09
	H48[c]	5.89 ± 0.04	6.20 ± 0.02
	H123	5.32 ± 0.07	5.62 ± 0.08
Pin1	H59	6.22 ± 0.27	5.57 ± 0.17
	H157	6.28 ± 0.16	6.84 ± 0.21

k_{cat}/K_M Values for Pin1 T152 Mutants		
Pin1 mutant	k_{cat}/K_M [mM^{-1} s^{-1}]	fold decrease
wild type	4669 ± 1032	1
T152A	14.3 ± 2.1	327
T152S	3003 ± 576	1.56

[a] pK_a and k_{cat}/K_M values are given as mean ± standard error of the mean (s.e.m.). [b] The substrates for Par14 and Pin1 were the peptides Suc-Ala-Arg-Pro-Phe-pNA and Ac-Ala-Ala-Ser(P)-Pro-Arg-pNA, respectively. Suc, succinyl moiety; pNA, *p*-nitroaniline; Ac, acetyl moiety. Standard abbreviations are used for amino acids. [c] H48 is located on the surface of Par14. This residue is not conserved among parvulins and shows titration behavior similar to that of free histidines.

Next, the pH titrations were repeated in the presence of model substrates. The peptidic ligands employed were very different in sequence and charge: Suc-ARPF-pNA and Ac-AApSPR-pNA were used for Par14 and Pin1, respectively. The difference in pK_a values of more than one pH unit (Table 1) for histidines H123 in Par14 and H157 in Pin1 can be explained by side-chain effects. Remarkably, the pK_a values of H42 of Par14 and the corresponding H59 of Pin1 converged in the presence of the peptide ligands. Residue H59 in Pin1 has been reported to be important for the catalytic activity.[13] These findings indicate very similar requirements for hydrogen-bonding during catalysis in these two types of small PPIases.

Our study represents the first crystallographic proof for rotamer positions and the tautomeric state of the two core histidines. Together with the microscopic pK_a values, these findings comply with a recent NMR study of *Staphylococcus aureus* PrsA by Heikkinen and colleagues,[10] who clearly showed hydrogen bonding between the two histidines. For the first time, this hydrogen-bonding network has now been shown to extend to threonine T118 for Par14. If the observed Cys/Asp-His-His-Thr motif represents a catalytic tetrad required for isomerase activity of parvulins, this very threonine should be essential for catalysis. To test this hypothesis, the corresponding T152A and T152S mutants of Pin1 were assayed for their activity (Figure 2A and Supplementary Figure 2), as the activity of Pin1 can be measured by means of an established and sensitive assay whereas that of Par14 cannot. While the Thr-to-Ser exchange only slightly decreased the activity, the T152A mutant displayed a more than 300-fold drop in catalytic activity (Figure 2B and Table 1). In line with this finding, alignments of parvulin family proteins showed Ser or Thr residues at this position in all members,[5,11,18] indicating that these two residue types are interchangeable.[12] S154 was previously believed to be crucial for activity in Pin1.[6] This position is filled by F120 in Par14.[7] Since only main-chain contacts to the supporting T118 are observed in our structure, the residue at this position can vary greatly in its side chain without affecting the stability or activity.

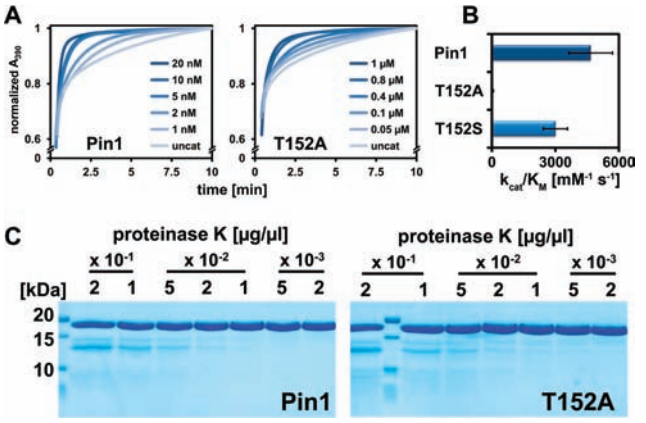

Figure 2. Assessment of the activity and stability of Pin1 T152 mutants. (A) The isomerase activity was determined for Pin1 wild-type and mutant proteins using the protease-coupled PPIase assay. The greatly differing protein concentrations used for these measurements should be noted. (B) k_{cat}/K_M values for wild-type Pin1 and the T152 mutants (mean ± s.e.m.) using Suc-ApSPF-pNA as the substrate. The value for wild-type Pin1 agrees reasonably well with the k_{cat}/K_M value of 3760 mM^{-1} s^{-1} reported previously for AApSPF-pNA.[13] (C) Limited proteolysis by proteinase K at the temperature of the enzyme assay. Equal amounts of protein were treated with the indicated amounts of proteinase K and incubated for 10 min. Clearly, wild-type Pin1 and the T152A mutant show very similar susceptibilities toward digestion by proteinase K.

As mentioned above, the two conserved histidines are considered to play a fold-stabilizing role.[14] Consequently, the measured large drop in activity for the T152A mutant could also be ascribed to reduced protein stability. To assess this hypothesis, we analyzed the protein stability of wild-type and mutant Pin1 proteins by limited proteolysis and thermal unfolding. In these experiments, the T152A mutant behaved nearly identically to the wild type toward limited proteinase K digestion (Figure 2C). Digestion with trypsin also resulted in very similar patterns for wild-type Pin1 and the T152A mutant (Supplementary Figure 3A), showing that Pin1 T152A is far less sensitive to protease than has been reported for the H59L mutant and the H59L/H157L double mutant.[14] Only the thermal unfolding behavior of T152A differed slightly in comparison with the T152S and wild-type proteins between 40 and 60 °C (Supplementary Figure 3B). These findings suggest that the T152A stability is rather unchanged under the employed assay conditions and thus that protein stability cannot be the cause for the mutant's dramatically decreased catalytic activity.

The Cys/Asp-His-His-Thr/Ser motif is strictly conserved in all parvulin sequences.[5,11,18] The only exception, which lacks the two histidines and a Cys/Asp moiety, is the SurA-like parvulin domain of *Escherichia coli* PpiD and, as expected, this protein does not show any isomerase activity.[19] Deposited parvulin and Pin1 structures show very similar orientations of the histidine side chains, although some of these contain modeled imidazole moieties in different ring-flipping states than suggested by our structure and the orientation reported by Heikkinen et al.[10] This probably has to be attributed to the fact that the limited resolution did not allow an unequivocal assignment. We conclude that the Cys/Asp-His-His-Thr/Ser motif is both conserved and required in the primary sequence and 3D structure of all small PPIases.

In summary, we have reported for the first time the direct observation of an extended hydrogen-bonding network with a backing threonine in small PPIases. This motif could function in precisely positioning and polarizing the Cys/Asp moiety for catalysis.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary Figures 1−3, Supplementary Table 1, and materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org. Coordinates and structure factors have been deposited in the Protein Data Bank as entries 3UI4 (Par14 refined to 0.8 Å resolution) and 3UI5 (rotating-anode sulfur SAD data).

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