Structural Analysis of the Mitotic Regulator hPin1 in Solution

INSIGHTS INTO DOMAIN ARCHITECTURE AND SUBSTRATE BINDING*

The peptidyl-prolyl cis/trans isomerase hPin1 is a phosphorylation-dependent regulatory enzyme whose substrates are proteins involved in regulation of cell cycle, transcription, Alzheimer’s disease, and cancer pathogenesis. We have determined the solution structure of the two domain protein hPin1(1–163) and its separately expressed PPIase domain (50–163) (hPin1PPIase) with an root mean square deviation of <0.5 Å over backbone atoms using NMR. Domain organization of hPin1 differs from that observed in structures solved by x-ray crystallography. Whereas PPIase and WW domain are tightly packed onto each other and share a common binding interface in crystals, our NMR-based data revealed only weak interaction of both domains at their interface in solution. Interaction between the two domains of full-length hPin1 is absent when the protein is dissected into the catalytic and the WW domain. It indicates that the flexible linker, connecting both domains, promotes binding. By evaluation of NOESY spectra we can show that the α1/β1 loop, which was proposed to undergo a large conformational rearrangement in the absence of sulfate and an Ala-Pro peptide, remained in the closed conformation under these conditions. Dissociation constants of 0.4 and 2.0 mM for sulfate and phosphate ions were measured at 12 °C by fluorescence spectroscopy. Binding of sulfate prevents hPin1 aggregation and changes backbone resonances across the active center and around the reactive and catalytically essential Cys13. In the absence of sulfate and/or reducing agent this residue seems to promote aggregation, as observed in hPin1 solutions in vitro.

Human Pin1 (hPin1)† is a key protein in post-phosphorylation regulatory mechanisms. It was originally identified in

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‡ The abbreviations used are: hPin1, human Pin1; CTD, C-terminal domain; DTT, dithiothreitol; HSQC, hetero-single-quantum coherence; NOESY, nuclear overhauser enhancement spectroscopy; PPIase, peptidyl-prolyl cis/trans isomerase; TOCSY, total correlated spectroscopy; PEG, polyethylene glycol; GST, glutathione S-transferase; TEV, tobacco etch virus; r.m.s.d., root mean square deviation.

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domain does. Binding affinities of full-length hPin1 and its isolated WW domain toward peptide substrates only differ in a factor of two, whereas hPin1_{PPIase} shows moderate affinity or no affinity toward them (13). In binding studies with cellular substrates of hPin1, the WW domain was shown to be responsible for interaction, and hPin1_{PPIase} does not bind any of the protein substrates (8). Interestingly, the optimal binding peptide for the WW domain WFPSPPLE (pintide) is most similar to the substrate WFPSPPR-pHnA, for which the highest PPIase activity of hPin1 (K_{d} = 20.mM^{-1} s^{-1}, unphosphorylated 170 mM^{-1} s^{-1}) has been measured (2).

Two crystal structures of full-length hPin1 are published and deposited in the Research Collaboratory for Structural Bioinformatics data bank (accession numbers: 1pin and 1f8a) (13, 14). In both structures WW and PPIase domains share a common interface. The WW domain and the catalytically active PPIase domain of hPin1 are connected by a glycine- and serine-rich stretch, which plays a yet unknown role in protein function. In crystal structures this linker seems to be flexible and forms no contacts to residues of the two folded domains.

The crystal structures suggest two possible conformations of hPin1. The first structure of hPin1 (1–163) co-crystallized with an Ala-Pro peptide (14), exhibits the “closed” active site of PPIase domain, including a phosphate-binding α/β loop, which is complexed to a sulfate ion and partly shelters the substrate binding motif, a proline ring pocket. The WW domain does not participate in substrate interaction, but together with the PPIase domain binds a PEG molecule close to the interface. In the second structure of hPin1 (1–163) a 70° rotation of the phosphate-binding loop leads to an “open” conformation of the PPIase domain active site (13). No sulfate ion is complexed to the structure. In that case, the phosphorylated peptide is bound exclusively to the WW domain, resulting in a twist of its β-sheet. Both structures are recognized as two different stages during the substrate-binding event. Sulfate is hypothesized to act as phosphoryl group mimetic, which, upon binding together with Ala-Pro dipeptide induces “closing” of the loop region.

To shed some light onto hPin1 domain interaction in solution and to investigate the proposed induced fit mechanism upon substrate binding, we solved the structure of full-length hPin1 (1–163) and of its PPIase (50–163) domain in complex with sulfate using nuclear magnetic resonance. Weak interaction of hPin1 domain was detected in the presence of the flexible linker in full-length hPin1, but no complex formation occurred after dissecting the protein into separated domains. NOESY techniques provide information about the integrity of the structure, indicating that the phosphate-binding loop remains in the closed conformation even in the absence of sulfate ions.

MATERIALS AND METHODS

Plasmid Construction—The coding DNA sequence for human Pin1 protein (RefSeq NM_006221) was used to search via BLASTN (15) in the dbEST-human data base at NCBI (www.ncbi.nlm.nih.gov) for corresponding EST clones and several of the clones with 5′-sequence identity (National Institutes of Health Image clones 110334, 171663, and 471113) were combined and used to amplify the Pin1 gene by PCR (forward primer: AATAAATAATCATACGTGCAAGGGAGGAAAGCTG (Ndel site underlined); reverse primer: AATAAATGATGATTATATTCTCACGTGGCGGAGGATGTG (EcoRI site underlined)). This PCR fragment was cloned into the NdeI-EcoRI sites of pET-28a vector to generate a protein fusion with N-terminal poly-His tag and thrombin site for purification of the protein. The DNA encoding the hPin1_{PPIase} (50–163) protein was constructed and amplified by PCR using the pET-28a, hPin1 gene and the following primers: forward, AATTTAAA- CATATGACATCATCAACACATCAATTGCGCGGAGGAGGAAAGCTG (Ndel site underlined) and reverse, AAATTTAATTTTTTATTTTCTCAGTGGCGGAGGATGTG (EcoRI site underlined). The PCR product was NdeI-EcoRI-ligated into the vector pET-41a (Novagen). The gene for hPin1_{PPIase} is preceded by a sequence encoding for the His_{6} tag. To receive the gene coding for the construct hPin1_{PPIase}-(6–39) PPIase using pET-28a, hPin1_{PPIase} was performed with the following primers: forward, AAATTAAATCATATCGAGCCAGCCCCTGAGGAGG (Ndel site underlined) and reverse, AAATTGAATTTATTGGCTG- GGGCCGGATGATGTTG (EcoRI site underlined). The PCR product was Ndel-EcoRI-ligated into the vector pET-42_mod_TEV, which was kindly provided by Dr. Axel Scheidig. The gene for hPin1_{PPIase} is preceded by a sequence encoding for glutathione S-transferase protein (GST), followed by a His_{6} tag and a tobacco etch virus (TEV) protease cleavage site.

Expression and Purification of Human Pin1, Pin1_{PPIase}, and Pin1_{WW}—All protein constructs were expressed in Escherichia coli strain BL21 (DE3) Codonplus RIL (Novagen). 50-ml overnight cultures of hPin1 or hPin1_{PPIase} were harvested and resuspended into 50 ml of M9 minimal medium enriched with either [^{15}N]ammonium chloride (1 g/liter) for uniformly labeling or with [^{15}N]ammonium chloride (1 g/liter) and [^{15}C]glucose (2.5 g/liter) for uniformly ^{15}N-/^{15}C-labeled (Cambridge Isotope Laboratory). Expression cultures were grown in isotope enriched minimal medium by inoculation of 1 liter with 50 ml of overnight-adapted cells. For unlabeled protein the 50-ml overnight culture was directly added to 1 liter of 2× YT (yeast extract tryptone) medium. After induction of protein expression at A_{600} = 0.4 with 1 mM isopropyl-1-thio-β-D-galactopyranoside, cells were shaken for a further 4 h at 37 °C, harvested, and centrifuged at 4 °C for 20 min at 5,000 × g in a Beckman J-2-HC centrifuge (Beckman Instruments, Palo Alto, CA). Cell rupture was performed using a Model 110S Microfluidizer (Microfluidics, Newton, MA) in 50 mM sodium phosphate or Tris/HCl buffer, pH 8.0, each containing 0.3 mM NaCl, 20 mM imidazole, and 2 mM β-mercaptoethanol supplemented with Complete™, EDTA-free, protease inhibitor mixture (Roche Applied Science, Penzberg, Germany). The cell lysate was ultracentrifuged at 4 °C in a Sorvall Discovery 100 centrifuge at 72,000 × g for 30 min. The supernatant was applied to a nickel-nitritolriacetic acid Superflow (Qiagen) column (2.5 × 20 cm), equilibrated with either 50 mM sodium phosphate or Tris/HCl buffer, pH 8.0, 0.3 mM NaCl, 20 mM imidazole. hPin1 and hPin1_{PPIase} proteins were eluted with an imidazole gradient of 20 to 200 mM in the corresponding buffer, concentrated, and washed in a Microspin microconcentrator (Filtron Technology Corp., Northridge, MA) with an exclusion size of 10,000 Da. The untagged GST fusion WW domain construct was expressed in 2× YT medium and purified by affinity chromatography with nickel-nitritolriacetic acid Superflow. The GST-His_{6} tags were removed by His-TEV protease (Invitrogen) at 4 °C directly on column. The untagged WW domain was in the flowthrough, whereas the GST-His_{6} tags and His-TEV protease remained bound to nickel-nitritolriacetic acid Superflow. Finally, the WW domain-containing fractions were pooled and concentrated in a Centricon tube with an exclusion size of 1,000 Da.

UV Spectroscopy—UV spectroscopy of the aggregation of hPin1 and all OD measurements were carried out using a CARY 100 Bio UV-visible spectrometer (Varian) equipped with a Peltier temperature control unit.

Fluorescence Titration Experiments—Fluorescence experiments were performed using an SLM Smart 8000 spectrophotofluorometer (Colora, Lorch, Germany) equipped with a PH-PC9635 photomultiplier. Sample concentration for hPin1_{PPIase} was 1 μM. The buffer contained 50 mM Tris/HCl, 1 mM DTT, pH 6.6, for measurements at 12 °C. For tryptophan fluorescence, samples were excited at 295 nm and the emission intensity was measured at 348 nm. The slit widths for the experiments were 1 and 16 nm. For buffer and volume effects, corrections were done by titration with blank buffer. Data were evaluated using the program Sigmoidal 7.0 by fitting a quadratic equation given by Müller et al. (16). In equilibrium one-to-one binding of a protein, P, and ion, I, to form a protein-ion complex, PI, can be expressed as in Equation 1.

\[ [P] + [I] \rightleftharpoons [PI] \]  

(Eq. 1)

The equilibrium dissociation constant, K_{d}, for this reaction is as follows,

\[ K_{d} = \frac{[P][I]}{[PI]} \]  

(Eq. 2)

where \( \langle P \rangle = [P] + [PI] \) and \( \langle I \rangle = [I] + [PI] \), \( K_{d} \) and \( [P] \) can be calculated from Equation 3.

\[ K_{d} = \frac{\langle P \rangle - [P]}{\langle I \rangle - [I]} \]  

(Eq. 3)

Substitution for [PI] leads to the following equation.
Structure Calculation—Structure calculation for hPin1 and the hPin1\_PPIase-sulfate complex was performed using the program CNS 1.0 (A. Brünger). High temperature torsion angle dynamics was performed at 50,000 K for 15 ps (1,000 steps) followed by a 15-ps cooling phase. In each case an ensemble of 100 structures was calculated from a random coil template. Ten models were selected on the basis of energetic criteria (low total energy, distance violations (NOE) < 0.2 \(\AA\) and dihedral angle violations < 5° using the accept.inp routine) to form a representative ensemble of the calculated structures. An average structure for each ensemble was generated, and in the case of hPin1 subsequent energy minimization was applied. All calculations were done on a Silicon Graphics Inc. Octane workstation. The program Sybyl (Tripos Associates, St. Louis, MO) was used for visualization, and the programs RasMol 2.6 (25), Molfacet 2.2 (26), and Raster3D (27) were used for figure production. The structures of hPin1 and hPin1\_PPIase-sulfate have been deposited in the Protein Data Bank (accession numbers 1NMV and 1NMW).

Error Determination—Errors of fitted \(K_0\) values and geometrical and energetic data were calculated by commercial programs (Sigmajit 7.0) and are defined as root mean square deviation,

\[
\text{r.m.s.} = \sqrt{\frac{\sum x_n^2}{n-1}} \quad (\text{Eq. 7})
\]

where \(x\) is the mean, \(\sigma\) a certain measured value, and \(n\) the total number of measured points.

RESULTS

The WW and PPIase Domains of hPin1 Interact Only Weakly in Solution—We determined the structure of full-length hPin1- (1-163) and its PPIase domain (hPin1\_PPIase- (50-163)) in phosphate buffer solution (50 \(\text{mM}\) Na\(_2\)SO\(_4\), 1 \(\text{mM}\) DTT, 5 \(\text{mM}\) EDTA), pH 6.6, and 27 °C using multidimensional nuclear magnetic resonance techniques. 1277 (1044) distance constraints, 78 (64) hydrogen bonds as well as 82 (74) \(\phi\) and 64 (52) \(\psi\) angle constraints were extracted from homo- and 13C,15N heteronuclear NOESY spectra, 15N HSQC spectra, and HNHA spectra of hPin1 and hPin1\_PPIase. 100 structures were calculated by torsion angle dynamics (CNS 1.0), and, on the basis of a set of exclusion parameters, an ensemble of 10 structures (Table I) was chosen to represent the final fold. Fig. 1 shows an overlay of the structures of full-length hPin1 fitted on either the WW domain (Fig. 1A) or the PPIase domain (Fig. 1B). Both domains could be determined with high precision resulting in low r.m.s.d. values over backbone atoms of 0.43 \(\text{Å}\) (WW) and 0.48 \(\text{Å}\) (PPIase), respectively. The sequence of hPin1 and the number of experimentally determined contacts, each residue is involved in, are presented in Fig. 1 (C and D). Although the WW and PPIase domain share a common interface in crystal structures, no distance constraints (NOEs) could be observed in any of the NOESY spectra between residues Ile\(_{28}\), Thr\(_{29}\), Asn\(_{30}\), and Ala\(_{31}\) and residues Asp\(_{136}\), Ala\(_{137}\), Ala\(_{140}\), Leu\(_{141}\), Ser\(_{147}\), and Gly\(_{148}\), all of which are proposed to be involved in domain interaction (13, 14).

A prerequisite for the presence of NOEs between atoms of these two domains is a relatively tight binding of the interacting partners. Lifetime of the complex should be long enough for NOEs to build-up. Consequently, distance constraints between binding partners are not observed in NOESY spectra of weakly bound and short lived complexes. A characteristic feature for weak interaction is the observation of high \(h_{\text{BB}}\) values. In such a case information on the interaction of binding partners can be obtained from chemical shift perturbation experiments. Binding can be monitored by changes in chemical shifts of resonances in NMR spectra. Spectra are collected in a way that the concentration of one of the proteins is stationary while concentration of the second protein is stepwise increased.

To investigate whether both domains of hPin1 interact in solution, we cloned and expressed the PPIase domain comprising residues Gly\(_{20}\) to Gln\(_{163}\) (hPin1\_PPIase) and compared the
chemical shifts of HN resonances in the $^{15}$N HSQC spectrum of hPinPPIase to the corresponding resonances in the $^{15}$N HSQC spectrum of full-length hPin1. As shown in Fig. 2, large chemical shift differences were observed of residues thought to form the proposed interface (black) and of neighboring amino acids.

The differences in chemical shifts between resonances of hPin1PPIase and the PPIase domain of full-length hPin1 indicate that interaction of domains in hPin1 occurs in solution along the interface observed in crystal structures. In contrast to the crystal structures (13, 14) equilibrium exists in solution within the intact hPin1 between a "bound" (complexed) and a "free" state, in which both domains are connected by a flexible linker but do not interact with each other. In solution the main populated conformation is the free state of hPin1.

**The Interdomain Linker Plays a Major Role in Promoting Domain Interaction**—A short flexible linker comprising Asn 40 to Gly49 connects both domains of hPin1. In crystal structures no contacts from amino acids of the linker exist to any other residues from either the WW or PPIase domain. Resonances corresponding to amino acids of this region are very weak or absent in $^{15}$N HSQC spectra, indicating that the linker is also flexible in solution. Consequently, no long or medium range
NOEs were found for residues Asn<sup>40</sup> to Gly<sup>29</sup>. To elucidate a possible role of the linker in domain interaction, we dissected the protein into its independent domains, excluding the linker region, to monitor their interaction by NMR. For this purpose we expressed the WW domain from hPin1 without the linker region, to monitor their interaction by titration. We dissected the protein into its independent domains, shown in Fig. 3.<sup>A</sup> The ratio of both proteins was reached (Fig. 3.<sup>E</sup>). Surprisingly, no shifts were observed after a 1:1 ratio of both proteins was reached (Fig. 3.<sup>E</sup>). The spectrum shown in Fig. 3.<sup>C</sup> (1:1 ratio) is only the sum of the spectra shown in Figs. 3.<sup>A</sup> (hPin1<sub>WW</sub>) and 3.<sup>B</sup> (hPin1<sub>PPIase</sub>). As can be seen from Fig. 3.<sup>E</sup>, the chemical shift differences observed in 15N HSQC spectra between resonances in the PPIase domain of full-length hPin1 (green) and hPin1<sub>PPIase</sub> (red) cannot be regained in the spectrum, in which the single domains are present in a 1:1 ratio (blue). These results demonstrate that no domain interaction occurs in the absence of the linker but that presence of the linker promotes complex formation.

**Structural Comparison of hPin1 in Solution and in the Crystal State**—Both crystal structures were determined in complex with small ligands, where either the active center of the PPIase domain is occupied by an Ala-Pro moiety and a PEG molecule is bound to the composite domain interface (14) or a phosphorylated peptide is bound to the WW domain (13). Minor differences in the active centers of the PPIase domains of crystal and solution structure may be direct consequences of these differences in the active centers of the PPIase domains of crystal and solution structure may be direct consequences of these different binding modes. There is evidence that the two highly conserved histidine ring systems (His<sup>157</sup> and His<sup>56</sup>) have different orientations in the three models. Whereas in the solution structure the rings are fixed by a number of distance constraints in a way that both His protons face each other (data not shown), the ring of either His<sup>157</sup> (14) or His<sup>56</sup> (13) is rotated by 180° around the preceding Cβ–Cγ bond in the crystal structures.

Because domains of hPin1 interact only weakly in solution, we had to compare both folds (WW and PPIase domain) separately to their corresponding domains in the crystal structures and to equivalent structures deposited in the RCSB data bank. The topology of the WW domain of full-length hPin1 is similar to those of many other WW domains solved so far. The program SSM (www.ebi.ac.uk/msd-srv/ssm) was used for a structural similarity search against the RCSB data bank and yielded high Z score values (6.5–10.4) and low r.m.s.d. values (<2.1 Å) along the C<sub>α</sub> trace to at least 20 other RCSB entries. Highest identity was found for hPin1<sub>WW</sub> (22) complexed to a Cdc25 peptide (1i6g, r.m.s.d. 1.22 Å) and to the WW domain (14) of the crystal structure of hPin1 (1pin, r.m.s.d. 1.39 Å). A lower convergence of 1.68 Å was found to the WW domain of the crystal structure (1f8a), complexed to a peptide isolated from the CTD of RNA polymerase II (13). In this structure the bound ligand induced a β-sheet twist. A data base search using the program DALI.
The loop is closed, the side-chain atoms of residue Ile 78 are stabilized by sulfate ions. The extended conformation of the α1/β1 loop region must be mutually exclusive. This hypothesis can explain why Pin1 slowly aggregates during spectra acquisition in the absence of sulfate ions within a time scale of hours. The extended conformation of the α1/β1 loop region, which either covers the active site of the PPIase domain (14) or sticks out from the core of the protein (13). The structure exhibiting the closed conformation is complexed to a sulfate ion ligated by residues Arg68, Arg69, and Lys63 (basic cluster) of the loop region (Fig. 4A, blue), whereas in the "extended" case, no such ion is present (Fig. 4B, cyan). Because the sulfate ion can mimic the phosphoryl group of a substrate molecule (14, 29), the two crystal structures are regarded as time-resolved snapshots of an induced-fit mechanism that facilitates substrate binding (15). Ranganathan et al. (14) suggest an obligatory interaction of substrate and the basic cluster during catalysis, based on the observation that hPin1 enzymatic activity against a tested set of peptide substrates has decreased upon addition of sulfate or phosphate. They propose that binding of substrates and these multivalent ions must be mutually exclusive. This hypothesis can explain why hPin1 slowly aggregates during spectra acquisition in the absence of sulfate ions within a time scale of hours. The extended loop comprises hydrophobic residues like Ile78 that might destabilize the protein and enhance protein aggregation. When the loop is closed, the side-chain atoms of residue Ile78 are alternatively packed into a core comprising side chain atoms of e.g. residues Pro70 and Lys63.

To investigate the proposed induced fit mechanism and binding affinities of sulfate and phosphate, we studied binding of hPin1PPIase to these ions by fluorescence titration experiments. Changes in the intrinsic fluorescence intensity of Trp73 of hPin1PPIase upon addition of sulfate and phosphate (Tris/HCl buffer) were used to estimate ion-binding affinity of the protein (Fig. 5). hPin1PPIase binds sulfate and phosphate with $K_d$ values of 0.4 and 2.0 mM, respectively (at 12°C). After proving ion binding, we recorded homo- and 15N heteronuclear NOESY spectra of hPinPPIase and/or hPin1 in the presence of 50–100 mM sodium sulfate and compared them to spectra acquired in the absence of sulfate ions. Surprisingly, almost all distance constraints observed in the presence of sulfate were also observed, when sulfate is lacking. As an example, most prominent NOEs between protons of the methyl group and of the H$_n$ of residue Ala116 and the N proton of Trp73 are shown in Fig. 6, where parts of NOESY spectra, recorded under different solvent conditions, are plotted.

Owing to our data the proposed induced fit mechanism does not occur in the hPin1 PPIase domain upon addition of sulfate or, what can be implied, upon addition of substrate molecules in solution. Although the loop region seems to be in the closed conformation in the absence of sulfate, flexibility of some residues changed on a small scale. Upon addition of sulfate, some resonances, which appeared as weak cross-peaks in 15N HSQC spectra recorded without sulfate, gained intensity and changed chemical shifts (e.g. Ser114).

Sulfate Ions Influence Surface Charges Around Residues of the Active Center—Next, we concentrated on the influence of sulfate ions on the structural integrity of the PPIase domain, because sulfate has a stabilizing effect on protein solubility. Therefore, chemical shift differences were measured between hPin1 in the presence and absence of sulfate. The positions of amide H$_n$ and N atoms and side-chain atoms, whose reso-
nances undergo chemical shift changes, are shown in Fig. 7 (A and B). Chemical shift changes are only observed for resonances of atoms in close proximity to the sulfate ion. Reasons for chemical shift changes upon ligand binding can either be structural changes, influencing the topological neighborhood of nuclei, or electrostatic shielding or deshielding effects, when charged groups of the ligand change the electronic environment of nuclei in the protein. In Fig. 7C chemical shift differences are plotted against the distance of amide groups to the sulfur atom of the ion. The black curve is fitted under the assumption that the chemical shift is straight proportional to the electric field strength (projected on the vector connecting the sulfur atom of the ion and Hx of the corresponding amide group) (28). In this case the data can be approximated by a 1/r2 function (black line). Considering experimental errors, resonance shifts observed upon sulfate binding seem to originate from a pure electrostatic effect and are less connected to a structural rearrangement.

As a control experiment, response of each amino acid of hPin1PPIase on the addition of sulfate was measured in an NMR titration experiment. Subsequent amounts of sulfate were added step-by-step to a 100 μM solution of the protein to reach a final ion concentration of 100 mM. All resonances of residues changing chemical shifts in the case of the PPIase domain of full-length hPin1, also showed chemical shift changes in the HSQC of hPin1PPIase. However, the absolute shift values were decreased to about 60–70% of the original values. Additionally, we observed shift changes of resonances of amino acids Glu65, Phe104, Glu105, and Thr143. From the titration experiment Kd values for sulfate binding at 27 °C could be obtained by plotting the concentration of sulfate ions against the chemical shift differences and fitting data points to a quadratic equation. In Fig. 8 fits of five amino acids are shown. The mean Kd value is ~7–8 mM.

After it could be ruled out that sulfate does induce structural rearrangement on a large scale, we had to look for a stabilizing effect caused by a change in the electrostatics of the protein. Fig. 9 shows GRASP surface charge representations of the sulfate ion binding loop and the active center of hPin1PPIase calculated either with (Fig. 9A) or without (Fig. 9B) sulfate ion electrostatics. Interestingly, the surface charges around the side chain of residue Cys113, a prerequisite for isomerase activity, change from strong positive (free form) to neutral (ion complexed form) upon ion binding. Because the thiol group of this cysteine (in contrast to Cys27) is involved in catalysis and is surface-accessible (30), the pKs value and, therefore, its reactivity might change, too. To investigate, whether Cys113 is responsible for aggregation we performed an experiment on relatively low concentration compared with our NMR conditions, where we followed aggregation by absorption spectroscopy monitored at 550 nm in a UV spectrometer. The aggregation behavior of 40 μM hPin1PPIase was followed in either Tris/HCl buffer solution or a solution where 50 mM sulfate or 1 mM DTT was added. In Fig. 10 the resulting A550 is plotted against time and fitted to a 1-exp function to trace the time course. Although, relative errors in data points are high, the time course of the sample with DTT closely resembles that of the sample where sulfate was added. Without one of these two substances aggregations seems to be faster. Thus, the stabilizing effects, induced upon sulfate binding, can be achieved by addition of reducing agent in the absence of sulfate.

**DISCUSSION**

We have solved the structure of the two domain peptidyl-prolyl cis/trans isomerase hPin1 in solution and shown that it differs from the structures determined by x-ray crystallography. Although chemical shift mapping was successfully applied to screen amino acids in the PPIase domain involved in binding...
The linker increases the local concentration of one domain around which does not interact with the rest of the molecule. The millimolar. Domain interaction is promoted by a flexible linker, seems to be in the range of hundreds of micromolar or even lower.

Titration of a 1M solution of Na2SO4 to yield a final concentration of 100 mM sulfate (27°C/H11005) or 13C HSQC spectra upon addition of sulfate are plotted. Titration curves of residues His64 (Kd 3.0 mM), Asp153 (Kd 18.1 mM), Glu131 (Kd 8.1 mM) are plotted. What is the reason for the differences observed in hPin1 domain interaction in the crystal state and in solution? In crystal structures protein is co-crystallized with either a substrate (13) or a xenobiotic PEG (14) molecule, both of which are complexed to the crystal both domains are tightly packed onto each other, whereas in solution this state is in exchange with another one, where no interaction between both domains occurs. Dissection to the WW domain, interdomain NOE constraints were absent in NOESY spectra. According to this observation the lifetime of the unbound state in full-length hPin1 is very short compared with the lifetime of the complexed state seems to be very short compared with the lifetime of the unbound state in full-length hPin1. Thus, the dissociation constant $K_d$ is dominated by a high $k_{off}$ value. In the crystal both domains are tightly packed onto each other, whereas in solution this state is in exchange with another one, where no interaction between both domains occurs. Dissection of the molecule and bringing together the separated domains does not lead to any observable complex formation in solution. Thus, the apparent $K_d$ value for interaction of the free domains seems to be in the range of hundreds of micromolar or even millimolar. Domain interaction is promoted by a flexible linker, which does not interact with the rest of the molecule. The linker increases the local concentration of one domain around the other (31). Assuming a linker length of 20 Å, the PPIase domain of hPin1 “senses” a local concentration of the WW domain of ~50 mM (and vice versa) compared with a concentration of 100 µM as used in our experiments for the separated domains.

What is the reason for the differences observed in hPin1 domain interaction in the crystal state and in solution? In crystal structures protein is co-crystallized with either a substrate (13) or a xenobiotic PEG (14) molecule, both of which are complexed to the composite interface region between the WW and PPIase domain. Titration of hPin1 in solution with increasing concentrations of PEG400 (Fig. 11) up to 3% (v/v) induces shifts in resonances of residues, which are at the PEG binding site of the WW in the crystal structure (14). It is very likely that binding of a substrate to the WW domain promotes domain interaction (32), a hypothesis that is summarized in Fig. 12. This hypothesis is in agreement with observations that hPin1WW can bind ligands in the absence of the PPIase domain, but binding is enhanced by a factor of 1.5–2 in the presence of the catalytic domain (13). Similar results were obtained when elucidating the structure of dystrophin (33). The WW domain of dystrophin cannot bind alone the dystroglycan ligand without the adjacent helical EF-hand-like domain. The two domains...
Solution Structure of hPin1

Fig. 11. A, Molscript representation of PEG binding site in the crystal structure (14). Residues, whose HN resonances undergo chemical shifts upon PEG addition, and PEG are represented by ball-and-stick models. B, part of a series of hPin1 HSQC spectra recorded with increasing PEG concentration (0, 0.1, 0.5, 1.0, 1.7, and 3.0%). In particular note the shift of Gln^33 as marked by an arrow.

Fig. 12. Model for the domain interaction in hPin1. In solution the equilibrium tends to the protein state with non-interacting domains. Upon addition of substrate the equilibrium is shifted toward the complexed form of hPin1.

actually form a composite recognition surface that is critical for the specificity to the substrate molecule.

The solution structure of the PPlase domain of hPin1 closely resembles the fold observed by Ranganathan et al. in the crystal state (14). Both structures are very similar and have only minor differences in the aI/b1 loop region. An extended conformation of this loop element was observed by Verdecia et al. (13) leading to the hypothesis of an induced-fit promoted rearrangement of the loop upon sulfate ion or substrate binding. By solving the structure of hPin1 and the PPlase domain in solution in the presence and absence of sulfate ions, we could demonstrate that no such structural rearrangement occurs, but the loop is in its closed conformation under both conditions. The open conformation observed in one of the crystal structures (13) might have its origin in crystallization conditions or crystal contact formation, but according to our studies, does not represent a snapshot of a substrate-receiving PPlase domain in solution. Nevertheless, some resonances of residues in the aI/b1-loop and amino acids in topological proximity to it gain intensity upon addition of sulfate. A change in the intensity of resonances in HSQC spectra was also observed for ArPin1 (29), which exhibits a similar phosphoryl-binding loop. The loop region seems to increase its rigidity when a phosphorylated substrate gets bound. This is not surprising taking into account that the sulfate ion or the phosphoryl moiety is trapped by flexible side chains of residues Arg^69, Arg^72, and Lys^60 of hPin1, which thereby undergo a loss of rotational freedom. Additionally, we performed a line width analysis of the HSQC spectra of hPin1 and the PPlase domain with and without sulfate (data not shown). Line width narrowing is observed in the N terminus (amino acids 1–6) and linker region (amino acids 39–49) of hPin1 caused by isotropic motion of the HN vectors. In contrast, only minor differences in the line width of residues of the loop region in the PPlase domain in the presence and absence of sulfate are found. The resonances of residues Arg^72, Trp^73, and Ser^114 gain intensity upon increasing ion concentration. These data are in agreement with results of T2 and HetNOE studies (32). Low HN resonance intensities in the absence of sulfate reflect the local flexibility of the corresponding amino acids. The flexibility of the resonances in the absence of sulfate causes fast motion of the HN vectors around the averaged protein backbone position with about 1–2 Å mean deviation, as can be seen in Fig. 1. If no sulfate or phosphorylated substrate is bound, residues Arg^69, Trp^73, and Ser^114 are flexible, because their amide groups are not involved in hydrogen bonds, whereas the motion of the sequential neighboring residues is still restricted. Sulfate binding induces formation of an ion- and water-based hydrogen network (14), including side-chain atoms of Lys^60 and backbone atoms of Arg^69, Trp^73, and Ser^114, which subsequently become more rigid. If we expected an opening of the loop, as observed in the crystal structure (13), more than 10 residues would be highly flexible and should undergo structural rearrangements of 5–28 Å. This would cause an isotropic movement of the corresponding HN vectors, and thus very low or negative HetNOEs and line width narrowing of all loop amide resonances should be observed.

The WW domain fold of the solution structure of hPin1 was found to have the highest identity to that of the NMR structure of hPin1_{WW} (1.2 Å, PDB code 1f6g) in complex with a Cdc25 peptide (22) and to the WW domain fold (14) within the crystal structure (1.39 Å, PDB code 1pin) published by Ranganathan et al. These findings might indicate that no substantial conformational changes occur in the small and compact structure upon ligand binding. Similar observations have been made by Wintjens et al. (22) on hPin1_{WW}, where only minor structural changes in the WW domain during substrate binding are reported. This is in contradiction to an observed β-sheet twist in the crystal structure of Verdecia et al. (13) where the WW domain is complexed to a CTD peptide. Because in the crystal structure (14) no substrate but a PEG molecule is complexed to the composite interface of the WW and catalytic domain, it is possible that the presence of both the peptide substrate and PPlase domain is a prerequisite for β-twist induction.

Samples of hPin1 and hPin1_{PPlase} diluted in phosphate or Tris/HCl buffer solutions in concentrations necessary for NMR structure determination showed severe aggregation within several hours at room temperature. Although NOESY spectra could be obtained under such conditions, new isotope-labeled samples had to be prepared for each triple resonance spectrum recorded. Thus, sulfate ions, which were reported to have a stabilizing effect on the PPlase structure of ArPin (29) and hPin1, were used to improve sample quality. Aggregation was not completely abolished under the influence of a 50–100 mM solution of sulfate ions, but it could be slowed down to enable acquisition over a period of several days.

The idea to use sulfate ions is based on two facts. First, sulfate ions mimic the binding properties of phosphoryl moieties of substrate peptides containing phosphorylated serine or threonine residues. Second, the crystal structure of hPin1 solved by Ranganathan et al. (14) is complexed to a sulfate ion, by side chains of arginine and lysine residues of the aI/b1 loop region. This loop is extended in the crystal structure of Verdecia et al. (13) solved in the absence of sulfate and presents hydrophobic residues to the bulk water. It was hypothesized that upon binding of sulfate large structural rearrangements of
the loop region are induced pushing the hydrophobic amino acids toward the core structure of the PPIase domain, thereby, increasing protein stability.

By comparing NOEs from the loop region of hPin1PPIase in NOESY spectra recorded in the absence and presence of sulfate, we could show that no significant changes in spectra occur. Only different chemical shifts of resonances were observed, but some resonances of $^{15}$N HSQC spectra gained intensity upon addition of sulfate (e.g. Ser$^{111}$). The conformational rearrangements observed in solution are minor and mainly influence the side chain of the ion chelating residues and the rigidity of amino acids in close proximity. The origin of chemical shift changes on sulfate addition is based on an electrostatic effect. Complexation of sulfate causes changes in surface charges around the active center. These alterations are probably the reason for changes in the intrinsic fluorescence signal of Trp$^{73}$ observed at 295 nm upon addition of sulfate ions. The increase in signal intensity can either be brought about by a gain in rigidity or a change in polarity of the environment, the indole ring is sensing. As can be seen from Fig. 9 polarity around Ser$^{72}$ and Trp$^{73}$ decreases upon sulfate binding, which might explain increase in fluorescence signal intensity.

The most dramatic changes in surface charges can be found at the complexation site (Arg$^{68}$, Arg$^{69}$, and Lys$^{66}$) and at the site of the active center, where amino acids His$^{157}$, His$^{59}$, and Cys$^{113}$ are located. It has been shown by mutation analysis that Cys$^{113}$ is important for cis/trans isomerase activity (14). Mutation of the cysteine to alanine or serine in hPin1 resulted in a 123- or 20-fold decrease in $K_d/K_m$, respectively. Residue Cys$^{113}$ is partly accessible by the attack of thiol group-modifying agents. Juglone, a Pin1 inhibitor, specifically attacks the thiol group and leads to a slow loss of structural integrity (30). The second cysteine (Cys$^{77}$) is buried in the interior and not accessible to, e.g., alkylating agents. Our studies show, that by adding the reducing agent DTT in millimolar quantities to a Tris/HCl buffer solution of hPin1 (after each spectrum acquisition was finished), we could slow down aggregation even in the absence of sulfate ions. One possible explanation for aggregation is that disulfide formation at Cys$^{113}$ occurs and leads to local unfolding of the protein. This in turn makes the second cysteine accessible for an attack and initiates oligomerization. An SDS-PAGE analysis of precipitated hPin1 without sulfate and DTT (data not shown) reveals the presence of dimers, trimers, and multimers, but only monomers were observed after reducing the sample. DTT prevents aggregation by reduction of disulfide bonds in hPin1.

How can we explain the stabilizing effect of sulfate? The reactivity of the protein thiol group depends on the accessibility of the thiolate group to the solvent, the fraction of thiol present as thiolate, and the intrinsic reactivity of the thiolate (basicity). In our case Cys$^{113}$ became less reactive upon addition of sulfate (Fig. 10). Cysteine residues in catalytically active sites often have low $pK_a$ values (35). One can speculate that the apparent $pK_a$ of Cys$^{113}$ increases (becomes more basic) upon addition of sulfate, making the residue less nucleophelic. Because the $pK_a$ defines the extent of ionization and reactivity at the given pH, an increase in the $pK_a$ should change the protonation state and induce shifts in the $\beta$-carbon or $\beta$-proton resonances. We could not follow the resonance shift of the Cys$^{113}$ $\beta$-proton; however, we found an upfield shift in the corresponding $\beta$-carbon resonance upon addition of sulfate (pH 6.8). In the absence of sulfate the thiolate may be stabilized by adjacent charged groups. Binding of sulfate changes the surface charges around Cys$^{113}$ from positive to neutral (Fig. 9), thereby destabilizing the thiolate ion and increasing the $pK_a$ of the residue. Most likely, disulfide formation is slowed down by this mechanism.

An interesting conclusion can be drawn from Fig. 7C concerning the electrostatic effect of sulfate. The electrical field introduced by the ion penetrates the protein core and influences amide proton and nitrogen chemical shifts within a radius of about 10–12 Å. A similar effect was observed in the phosphorylated form of the protein huridin, when the phosphate moiety was titrated from a monoanionic to a dianionic state (34). Here, changes in hydrogen bonds and chemical shifts occurred within a radius of 10 Å. Based on this two observations we can suggest that the cut-off value for electrostatic contribution to energy functions used for the calculation of protein structures, protein dynamics, and molecular modeling procedures has to be at least 10 Å to include the full electric field effect.

Because sulfate is regarded as an analogue for the phosphoroly moiety of a substrate peptide of hPin1 in vitro, we have to elucidate its role in vivo. Do sulfate or phosphate ions in cells play a possible regulatory role in hPin1 function? Competition between phosphate ions and non-phosphorylated tetra-peptides was found in in vitro assays, when the phosphate:substrate ratio was higher than 2000 (14). The dissociation constants obtained by fluorescence spectroscopy at 12 °C are 0.4 mM for sulfate and 2 mM for phosphate, respectively. The $K_d$ values increase upon raising temperature, because it has been measured for sulfate in a NMR titration experiment (7–8 mM at 27 °C). From these experiments we can estimate that the dissociation constants for both ions in cells at 37 °C are around 20–50 mM. The cellular concentrations of free sulfate and free phosphate ions are found to be 1 (total concentration was 10 mM, including bound ions) and 2–5 mM (total concentration, 50–60 mM, including bound ions), respectively. Assuming that the concentrations of hPin1 (36) and substrate molecules in cells reach micromolar values and the binding affinities of hPin1 to phosphorylated protein substrates are at least 10 times higher than for non-phosphorylated tetra-peptides ($K_d > 500 \mu M$) measured in a previous study (14), competition of multivalent ions for the phosphoryl binding site in hPin1 is negligible. Thus, it is unlikely that sulfate and phosphate ions play a regulatory role in hPin1 function in vivo.

What is the biological and pharmaceutical implication of our studies on hPin1? hPin1 is of great interest for cancer therapy and inhibition of its activity might prevent mitosis and, thus, malignant cell division. Bearing the crystal structure in mind and looking from a drug engineer’s view onto this molecular target, one might have two options for efficient development of a new anti-cancer drug. Inhibitors of hPin1 activation can either be addressed against the catalytic center of the PPIase domain or against the phospho-peptide binding site of the WW domain. Our model, describing a more dynamic domain interaction, offers a third strategy for rational drug design. Drugs preventing the formation or untying of a common binding interface might influence hPin1 function, too. Although we do not know yet how essential these dynamics are for hPin1 function in vivo, inhibiting domain interaction might prevent the protein from binding and targeting receptor molecules. Searching for “interface drugs” seems to be very promising, because it has already been shown by co-crystallization with PEG (14) that xenobiotic molecules of low molecular weight might induce interface formation.

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