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# Flexibility in the PP1:spinophilin holoenzyme

**Michael J Ragusa**[a], **Marc Allaire**[b], **Angus C. Nairn**[c], **Rebecca Page**[a], and **Wolfgang Peti**[d,*]

[a]Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI, USA

[b]National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY, USA

[c]Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA

[d]Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI, USA

## Abstract

Protein phosphatase 1 (PP1) interacts with ~200 regulatory proteins to form holoenzymes, which target PP1 to specific locations and regulate its specificity. While it is known that many PP1 regulatory proteins are dynamic in the unbound state, much less is known about the residual flexibility after PP1 holoenzyme formation. Here, we have used small angle X-ray scattering to investigate the flexibility of the PP1:spinophilin holoenzyme in solution. Collectively, our data shows that the PP1:spinophilin holoenzyme is dynamic in solution, which allows for an increased capture radius of spinophilin and is likely important for its biological role.

### Keywords

PP1; spinophilin; small angle X-ray scattering; flexibility; BILBOMD

## Introduction

Protein Phosphatase 1 (PP1) is a key serine/threonine phosphatase for regulating numerous essential cellular events including glycogen metabolism, neuronal signaling and protein synthesis [1]. However, PP1 itself exhibits very little substrate specificity. Instead, specificity is achieved by its interaction with ~200 different regulatory proteins that associate with PP1 to form highly specific holoenzymes [2]. Interestingly, PP1 regulatory proteins are often highly dynamic and lack a common 3-dimensional fold in their unbound forms, and thus belong to the class of proteins known as intrinsically unstructured proteins [3–5]. This flexibility is vital for their biological functions, as it allows them to interact through extensive interaction surfaces with PP1, where they commonly bind with significantly reduced flexibilities [4,6]. However, some regulators retain a significant degree of flexibility even after binding PP1 [6,7]. For example, the residual flexibility upon binding PP1 is essential for the proper regulation of PP1 by Inhibitor-2 [7]. Currently, the number of



*Corresponding Author, wolfgang_peti@brown.edu.

PP1 regulatory proteins with residual flexibility when bound to PP1, as well as the role of this flexibility in their biological functions, is unclear.

Spinophilin is a multi-domain scaffolding protein that targets PP1 to the post synaptic density (PSD) through its interaction with F-actin [8]. In the PSD, the PP1:spinophilin complex is additionally targeted to AMPA receptors via its PDZ domain, which is immediately C-terminal to the PP1-binding domain [9]. Once localized, the holoenzyme dephosphorylates Ser845 on the GluR1 subunit of AMPA receptors thereby regulating long term depression, a process critical for learning and memory formation [10].

Recently, we determined the 3-dimensional structure of the PP1:spinophilin holoenzyme [4]. Although the spinophilin PP1-binding domain is intrinsically unstructured in its unbound state, it folds upon binding to PP1 into a single, stable conformation. Notably, in the crystal, two molecules of the PP1:spinophilin holoenzyme were present in the asymmetric unit [4]. Interestingly, the structure of the spinophilin PP1 binding domain is identical between the two molecules in the asymmetric unit. In contrast, strong continuous electron density was only observed for one of the spinophilin PDZ domains. The fact that essentially no electron density was observed for the second PDZ domain, suggests that it was dynamic in respect to the spinophilin PP1-binding domain in the crystal. This also suggests that the residues connecting the spinophilin PP1-binding and PDZ domains are flexible, allowing the two domains to rotate independently of one another. Furthermore, the first, ordered spinophilin PDZ domain forms extensive crystal contacts with a PP1 symmetry mate, and thus crystal packing also likely contributes to the additional reduced flexibility between the spinophilin PP1-binding and PDZ domains (Fig. 1). Thus, to investigate the flexibility and structure of the PP1:spinophilin complex in solution, we collected small angle X-ray scattering (SAXS) data.

## 2. Materials and Methods

### 2.1. Protein expression and purification

$PP1\alpha_{7–330}$ and $spinophilin_{417–583}$ were expressed as described [4]. The $PP1\alpha_{7–330}$:$spinophilin_{417–583}$ complex was purified using a previously described protocol [4] with the following changes. After elution from Ni-NTA resin (Qiagen), the PP1:spinophilin complex was purified using a Superdex 200 26/60 size exclusion column (GE Healthcare) equilibrated with PP1 complex buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM TCEP). Tobacco Etch Virus protease (TEV) was added to cleave the $His_6$-tag from $PP1\alpha_{7–330}$. After digestion was complete, subtraction purification was performed using Ni-NTA resin (Qiagen) for the removal of TEV and the cleaved $His_6$-tag. In the final purification step, the complex was purified using a Superdex 75 26/60 size exclusion column (GE Healthcare) equilibrated with PP1 complex buffer. Fractions containing protein, as verified by SDS-PAGE, were pooled and stored at 4°C.

### 2.2. SAXS measurements

The PP1:spinophilin holoenzyme samples used for all SAXS measurements was produced within 24 hours of data acquisition and stored at 4°C. Immediately prior to the SAXS experiments, the sample was concentrated to either 0.5 mg/ml or 0.9 mg/ml at 4°C and filtered through a 0.02 μm filter (Whatman). Synchrotron X-ray scattering data were collected at the National Synchrotron Light Source (NSLS) beamline X9. SAXS data were collected using a MarCCD 165 located at 3.4 m distance from the sample. Wide angle X-ray scattering (WAXS) data were collected simultaneously with SAXS data using a Photonic Science CCD located at 0.47 m from the sample. 20 μl of sample was continuously pushed through a 1 mm diameter capillary for 180 s of measurement time and exposed to a 400 ×

200 microns X-ray beam. Scattering data for the complex was collected at concentrations of 0.5 mg/ml and 0.9 mg/ml. Normalization for beam intensity, buffer subtraction and merging of the data from both detectors were carried out using PRIMUS [11]. Theoretical scattering for the PP1:spinophilin crystal structure was calculated using FOXS [12]. A Guinier approximation, $I(q) = I(0)\exp(-q^2Rg^{2/3})$, where a plot of $\ln(I(q))$ and $q^2$ is linear for $q<1.3/R_g$, was performed on four independent scattering trials and averaged to determine the radius of gyration [13]. GNOM was used to determine the pair distribution function [P(r)] and maximum particle dimension ($D_{max}$) [14]. HYDROPRO was used to calculate the $R_g$ and the distribution of distances for the PP1:spinophilin crystal structure for direct comparison with the $R_g$ from the Guinier approximation and P(r), respectively [15]. The linearity of the Guinier region and the forward scattering intensity were used to validate that the PP1:spinophilin complex sample was monodisperse in solution. The forward scattering intensity, I(0), is the theoretical scattering at a q value of 0 and is proportional to the molecular weight of the sample [16]. I(0)/c, where c is concentration, was identical for all PP1:spinophilin measurements, demonstrating that the molecular weight of the complex does not change with increasing protein concentration.

### 2.3. BILBOMD

BILBOMD [17] was used to investigate the flexibility of the PP1:spinophilin complex. Molecular dynamics (MD) simulations in BILBOMD were used to generate 12,000 structures with a $R_g$ range of 20 – 50 Å (200 structures/Å; 2 calculations) [17]. Theoretical scattering curves were calculated for each structure using FOXS and compared to the experimental SAXS data [12]. The single best fit structure is defined as the structure with the lowest discrepancy ($\chi^2$) between the theoretical and experimental data. A minimal ensemble (MES) model was generated as previously described [17]. The MES was selected as the best model for the PP1:spinophilin complex in solution. The program DynDom was used to determine the spinophilin PDZ domain rotation between the crystal structure and the MES [18].

## 3. Results

### 3.1 The PP1:spinophilin complex is more extended in solution

Theoretical scattering data based on the PP1:spinophilin crystal structure and experimental scattering data correlate poorly with a discrepancy value ($\chi^2$) of 3.70. This shows that the complex adopts a different conformation in solution than in the crystal (Fig. 2a). Similarly, the radius of gyration ($R_g$) of the PP1:spinophilin complex in solution (29.1 ± 0.4 Å, as determined by the Guinier approximation) is 3.4 Å larger than the $R_g$ calculated from the crystal structure (25.7 Å) (Fig. 2a). We determined the pair distance distribution function P(r), which is the distribution of all interatomic distances in the PP1:spinophilin complex, using GNOM (Fig. 2b). Not surprisingly, the P(r) of the PP1:spinophilin holoenzyme also shows that the complex is elongated in solution with a $R_g$ of 29.05 Å and a maximal dimension of 90 Å. The agreement between the Guinier derived $R_g$ and P(r) derived $R_g$ is excellent, as expected for high-quality SAXS data. A comparison of the PP1:spinophilin solution P(r) with the distribution of distances in the PP1:spinophilin crystal structure, as determined using HYDROPRO, is shown in Fig. 2b. The overall shape of the PP1:spinophilin P(r) is conserved between the crystal structure and the solution data. However, the P(r) function for the crystal structure falls to zero at shorter distances than the solution P(r). Taken together, the differences observed in the $R_g$ and P(r) between the PP1:spinophilin holoenzyme solution and crystal data demonstrates that the PP1:spinophilin complex adopts a more extended structure in solution.

### 3.2 BILBOMD analysis

To further investigate the conformation of the PP1:spinophilin holoenzyme in solution, we used the program BILBOMD. BILBOMD samples conformational space using molecular dynamics (MD) simulations and then selects for the models that have the best agreement between the theoretical and experimental scattering data [17]. The PP1:spinophilin crystal structure (PDBID:3EGG) was used as a starting model for the MD simulations. Spinophilin residues 490–494, which form a linker between the PP1-binding and the PDZ domain and for which no electron density was observed in one molecule in the asymmetric unit of the PP1:spinophilin crystal, were defined as flexible. PP1 and spinophilin residues 424–489 were defined as a single fixed entity in space, while the spinophilin PDZ domain was defined as a rigid body that was allowed to move with respect to PP1. Despite defining a $R_g$ range of 20–50 Å for the simulations, only structures with a $R_g$ between 23 and 32 Å were sampled in the MD calculations, showing a limited flexibility between the spinophilin PP1-binding and PDZ domain (Fig. 3a). The single best fit model has a $R_g$ of 27.0 Å. The theoretical scattering profile for this model is in good agreement with the experimental scattering data ($\chi^2 = 1.30$), a significant improvement over the crystal structure fit ($\chi^2 = 3.70$) (Fig. 3b).

### 3.3 Minimal ensemble model

A minimal ensemble model (MES) was created that includes the single best fit model (73%) as well as two additional models (15% and 12%). The MES does not improve the overall fit to the experimental scattering data, but it demonstrates that an ensemble of structures fits the data equally well ($\chi^2 = 1.30$). In the MES model, the spinophilin PDZ domain is ~18 Å extended away from the PP1 hydrophobic substrate binding groove and rotated by ~140±10° relative to the position of the PDZ domain in the crystal structure (Fig. 4a). All three structures that form the MES are related by a rotation around the flexible spinophilin linker (residues 490–494) without a significant movement of the PDZ domain relative to PP1 (Fig. 4b and c). The most likely reason for the MES $\chi^2 \geq 1$ fit is the flexible C-terminal tail of PP1 (residues 300–330), which was present throughout all SAXS experiments, but not used in the BILBOMD calculations.

## 4. Discussion

Using SAXS, we have demonstrated that the PP1:spinophilin complex adopts a significantly more extended conformation in solution than in the crystal structure, where this extension is restricted by crystal packing. The extended solution structure is the result of a flexible linker between the spinophilin PP1-binding and the PDZ domain (residues 490–494). This allows the spinophilin PDZ domain to move relative to the PP1-binding domain, which becomes fixed in a single conformation upon PP1 holoenzyme complex formation. Since the theoretical scattering data of the MES model and the experimental scattering data agree very well, the dynamics within the PP1:spinophilin complex is likely restricted to the flexible linker connecting the spinophilin PP1-binding and PDZ domains.

The flexibility between the spinophilin PP1-binding and PDZ domains is important for three reasons. First, the BILBOMD MES ensemble structure demonstrates that the spinophilin PDZ domain does not extend any of the three recognized substrate binding grooves on PP1. Thus, the PDZ domain will not create an additional binding site for PP1:spinophilin holoenzyme substrates. This is in excellent agreement with biochemical data, which showed that the spinophilin PDZ domain does not enhance binding to PP1 or play a role in substrate recognition [9,19]. Second, our results suggest that the spinophilin PDZ domain acts independently from the spinophilin PP1-binding domain, despite the short four residue linker. This provides further corroboration that the PDZ domain likely behaves solely as a

targeting domain. Third, the four residue flexibility in the PP1:spinophilin holoenzyme will increase the capture radius of the spinophilin PDZ domain for its biological targets, for example, the GluR2/3 subunits of the AMPA receptor. The increased capture radius likely allows for more efficient targeting of the PP1:spinophilin holoenzyme *in vivo*.

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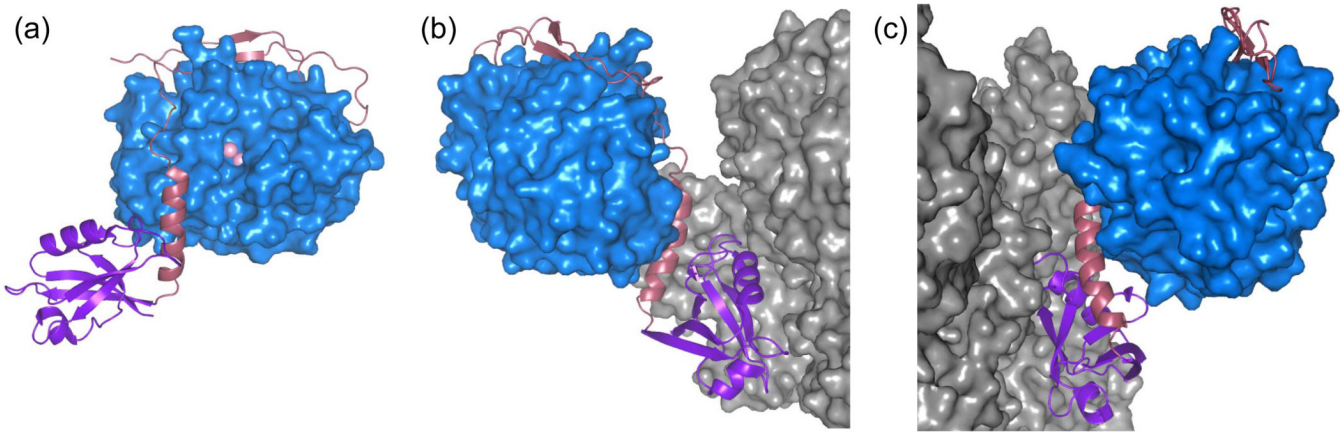


**Fig. 1.**

a: The PP1:spinophilin holoenzyme structure (PDB ID: 3EGG): PP1 (blue surface), spinophilin PP1-binding domain (red, cartoon), spinophilin PDZ domain (purple, cartoon). b: Two PP1:spinophilin symmetry mates are shown as gray surface representations to highlight the crystal packing around the spinophilin PDZ domain. c: 135° rotation of b.

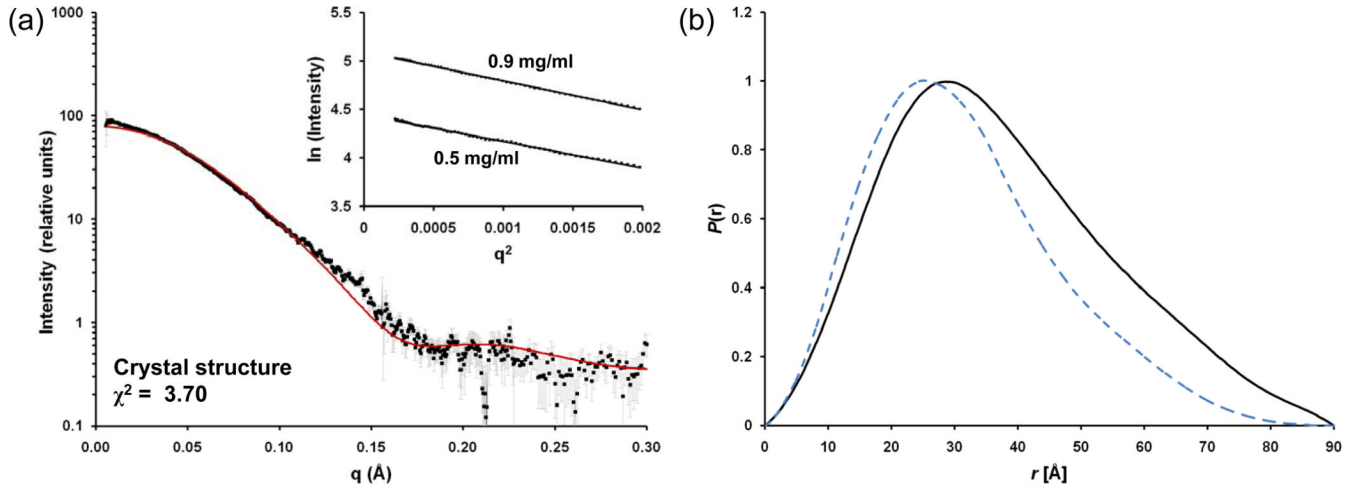


**Fig. 2.**

a: SAXS data for the PP1:spinophilin holoenzyme is shown as black squares with error bars as grey lines. Guinier plots for 0.5 mg/ml and 0.9 mg/ml are shown as an inset. b: The P(r) of the PP1:spinophilin complex from SAXS data (black line) and based on the crystal structure (blue, dashed line).

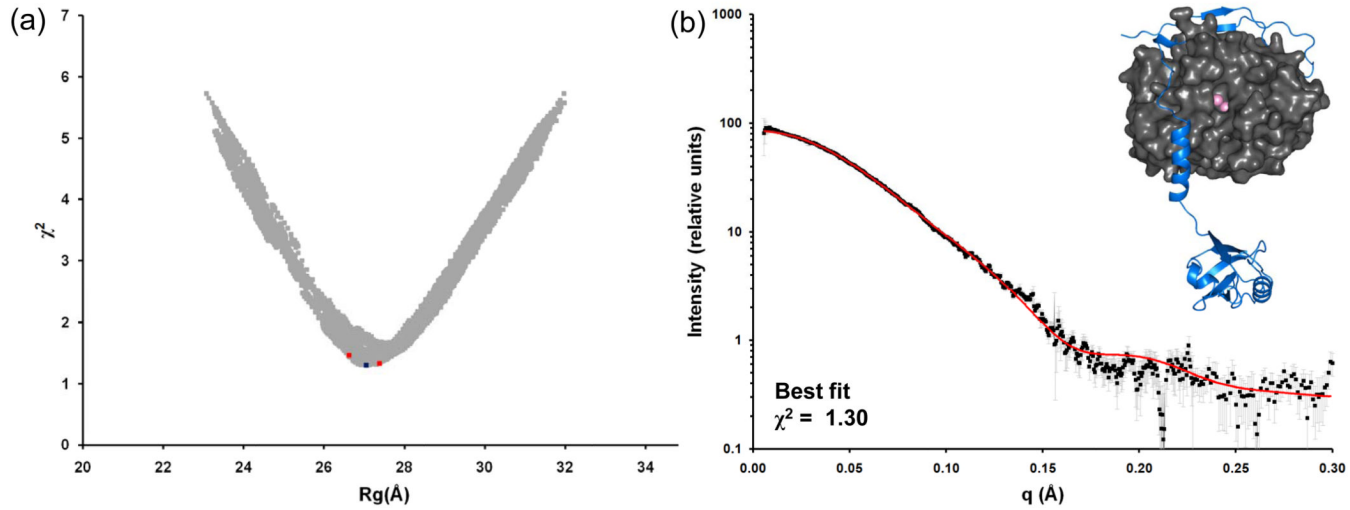


**Fig. 3.**

a: $R_g$ for each BILBOMD conformer plotted against the $\chi^2$. b: Comparison of the theoretical scattering of the single best fit model (red line) and the experimental data (black squares).

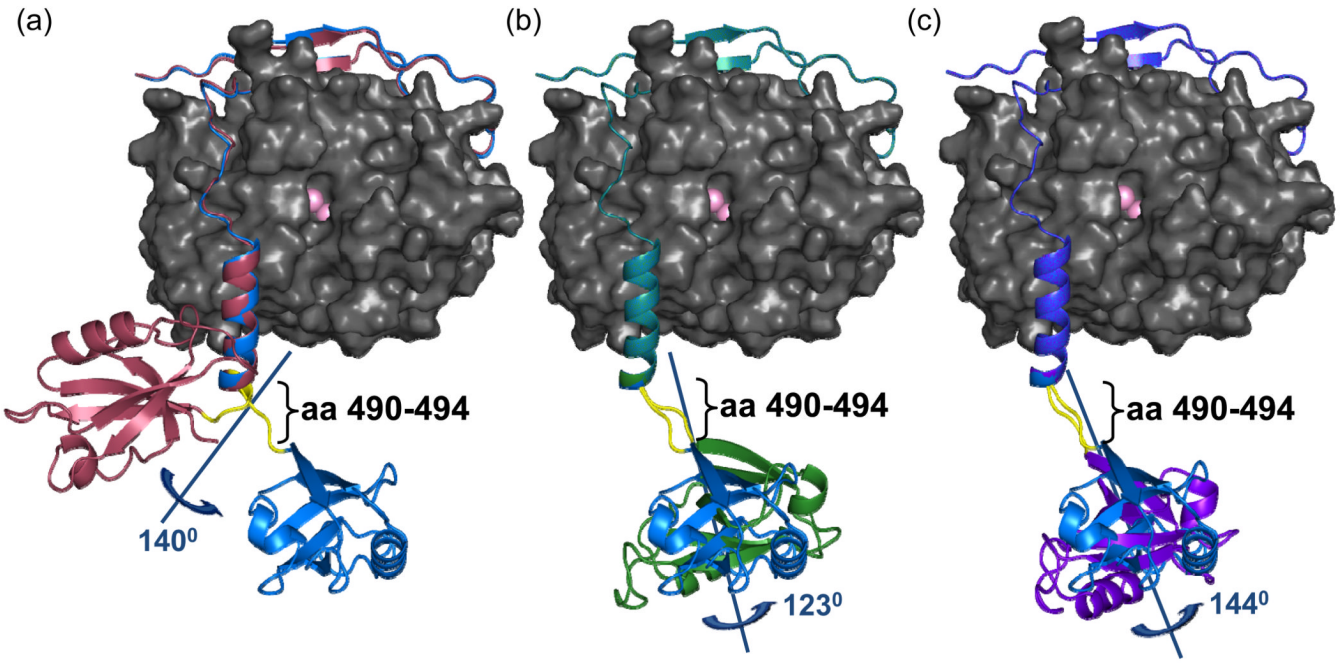


**Fig. 4.**

a: Comparison of the structure of spinophilin in the crystal structure (red) and the single best fit structure from BILBOMD (blue). The flexible linker of spinophilin (residues 490–494) is colored in yellow. b–c: A comparison of single best fit structure (blue) with the two additional conformers which form the MES model (green and purple). The axis of rotation is shown as a blue line and the angle of rotation is indicated.