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Pan1 is an intrinsically disordered protein with homotypic interactions

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Abstract

The yeast scaffold protein Pan1 contains two EH domains at its N-terminus, a predicted coiled-coil central region, and a C-terminal proline-rich domain. Pan1 is also predicted to contain regions of intrinsic disorder, characteristic of proteins that have many binding partners. In vitro biochemical data suggest that Pan1 exists as a dimer, and we have identified amino acids 705–848 as critical for this homotypic interaction. Tryptophan fluorescence was used to further characterize Pan1 conformational states. Pan1 contains four endogenous tryptophans, each in a distinct region of the protein: Trp³¹² and Trp⁶⁴² are each in an EH domain, Trp⁹⁵⁷ is in the central region, and Trp¹²⁸⁰ is a critical residue in the Arp2/3 activation domain. To examine the local environment of each of these tryptophans, three of the four tryptophans were mutagenized to phenylalanine to create four proteins, each with only one tryptophan residue. When quenched with acrylamide, these single tryptophan mutants appeared to undergo collisional quenching exclusively and were moderately accessible to the acrylamide molecule. Quenching with iodide or cesium, however, revealed different Stern-Volmer constants due to unique electrostatic environments of the tryptophan residues. Time-resolved fluorescence anisotropy data confirmed structural and disorder predictions of Pan1. Further experimentation to fully develop a model of Pan1 conformational dynamics will assist in a deeper understanding of the mechanisms of endocytosis.

Keywords

Pan1; intrinsically disordered protein; EH domain; tryptophan fluorescence quenching; time-resolved fluorescence anisotropy

INTRODUCTION

Clathrin-mediated endocytosis (CME) is a sequential process that requires the coordination of roughly 60 different proteins to bring extracellular material or plasma membrane-localized transmembrane proteins into the cell. Distinct cohorts of cytosolic proteins with different functions arrive sequentially at foci on the plasma membrane to support a nascent endocytic pit as it matures into a fully formed endocytic vesicle. These proteins have a variety of functions, including binding transmembrane cargo proteins, lipid, and coat components, as well as stimulating cytoskeletal machinery for vesicle internalization. CME is well conserved in all eukaryotic cells, and valuable lessons can be drawn from studies of endocytic proteins from the yeast *Saccharomyces cerevisiae*.

Many of the proteins involved in CME have been well characterized structurally. For instance, the structure and function of the clathrin coat has been described extensively^{1,2},

and certain adaptor proteins have domains with solved structures, such as the EFC/F-BAR of the yeast Syp1³ or the ENTH domain of the yeast epsins Ent1/2^{4,5}. However, other protein components are only understood genetically or biochemically, due to an inability to solve their structures using crystallography, caused in part by the presence of intrinsically disordered regions in these proteins. Recent studies have implicated intrinsically disordered proteins as critical in signaling pathways, specifically those that involve endocytosis^{6,7}. For instance, yeast Abp1 and Las17 are predicted to have intrinsically disordered regions⁷, and the unstructured C-terminus of mammalian AP180, punctuated by small beta turns, acts to recruit clathrin to endocytic sites⁸.

Pan1 is an essential yeast protein that provides a scaffold for CME by interacting with both early- and late-acting endocytic factors. It has numerous binding partners and contains multiple predicted regions of disorder; these are both characteristics of a “hub” protein, and provide a framework for understanding the role of Pan1 in regulating endocytosis⁷. At its N-terminus, Pan1 contains two Eps15 Homology (EH) domains, which have classically been characterized as binding partners to asparagine-proline-phenylalanine motifs (NPF) found in endocytic adaptors. Specifically, Pan1 binds Yap1801 and Ent1, both adaptor proteins that contain multiple NPF motifs^{9,10}. At its C-terminus, Pan1 stimulates Arp2/3-dependent actin polymerization that is required for scission and internalization of the endocytic vesicle¹¹. Additionally, Pan1 binds the type I yeast myosins, Myo3 and Myo5, which are required for the internalization step^{12,13}. While Pan1 is a critically important protein to endocytosis, the mechanism of its function at endocytic sites and what guides its sequential interactions with binding partners are poorly understood.

This study aims to further the understanding of Pan1 function as an endocytic scaffold through biophysical and biochemical analyses. Chromatography and velocity sedimentation of the central domain revealed that it is a dimer in vitro, and we mapped the amino acids that are critical for this homotypic interaction. To obtain conformational information about Pan1 without the benefit of crystal structures, we used spectroscopic techniques capitalizing on the intrinsic fluorescence of tryptophan. The Pan1 protein has four endogenous tryptophans, each in a functionally significant region, and through quenching experiments and time-resolved anisotropy using single tryptophan mutants, we determined the accessibility of these tryptophans and gained insight into Pan1 architecture. This work continues the characterization of Pan1 as a hub protein with long regions of intrinsic disorder that will allow for a full understanding of Pan1 function during endocytosis.

METHODS

Plasmid creation

Recombinant proteins with GST-, -TAP, and His₆- tags were created using primers containing homologous regions to both Pan1 and the plasmid backbone. Both plasmids for protein expression in yeast (pEG(KT)) and bacteria (pET28a) were linearized with restriction enzymes, purified via gel extraction (Qiagen), and transformed into W303 diploid yeast with the PCR product generated from the primers above. The resulting plasmids were then sequenced (GeneWiz) and transformed to bacterial expression strains (Rosetta) or a protease resistant yeast strain (BJ2168).

Site-directed mutagenesis

Mutagenesis of the tryptophans in Pan1 and the Tandem Affinity Purification (TAP) tag was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Silent mutations were added for analysis by restriction digest. Mutations were created in bacterial plasmids containing Pan1 fragments and full-length Pan1 was

reconstituted in the pEG(KT) plasmid through PCR and homologous recombination. For tryptophan fluorescence experiments, the tryptophan in the calmodulin binding peptide was replaced with a phenylalanine using site-directed mutagenesis, and this did not disrupt calmodulin binding.

Bacterial protein purification

Protein fragments were recombinantly-tagged with His₆ via homologous recombination using a modified pET28a vector containing yeast ARS and URA3 selection³. Bacteria containing the expression plasmid were grown in 40 mL LB with appropriate selection and 2% glucose to saturation, added to 360 mL SB and incubated at 37°C for 1 hour, and induced with 0.3 mM isopropyl-β-D-thio-galactoside (IPTG) for 6 hours at 30°C. Bacterial cells were pelleted, slow frozen to −20°C, lysed with an incubation of 20 mg lysozyme and sonication, and centrifuged at 26000 g for 20 minutes at 4°C. The supernatant was bound to 1mL of equilibrated Talon metal affinity resin beads (Clontech) with 5 mM imidazole, washed with 20 mM and 30 mM imidazole, and eluted with 500 mM imidazole. The imidazole was removed using a P-10 desalting column (GE Healthcare) and the protein was concentrated with 30K Amicon Ultra centrifugal filtration devices (Millipore) as necessary.

Yeast protein induction and harvesting

For purification of yeast proteins, fragments were cloned into the pEG(KT) plasmid as described previously¹⁴. Plasmids were transformed into a protease-deficient yeast strain (BJ2168) and the yeast was grown in 1.0 L of YNB media lacking uracil and leucine in the presence of 2% raffinose. Once an optical density of 1.0 to 2.0 at 600 nm was reached, the yeast were induced with 2% galactose for 12–16 hours. Yeast cells were harvested via centrifugation (2700 g for 10 minutes), washed with cold water, and centrifuged again. The resulting yeast pellet was resuspended in a minimum amount of cold water, 1X 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) hydrochloride (Fisher Scientific) was added, and poured dropwise into liquid N₂. The frozen yeast pellets were then placed in liquid N₂ in a Waring blender and blended until the yeast was a fine powder, and stored at −20°C.

Yeast protein purification

Pan1 protein with a C-terminal TAP-tag was purified using IgG- and CaM-sepharose beads (GE Healthcare). Yeast powder (~2–3 g) containing the TAP-tagged protein was thawed to 10 mL with 1X TBS (50 mM Tris-HCl pH 7.5, 100 mM NaCl), 2 mM DTT, and 100 μL protease inhibitor cocktail for fungal and yeast extract (Sigma). The lysate was rotated at 4°C for 30 minutes and centrifuged at 26000 g for 10 minutes at 4°C and 186000 g for 30 minutes at 4°C. The resulting supernatant was incubated with 1.0 mL of 50% slurry of equilibrated IgG-sepharose beads (GE Healthcare) for 8 hours. After binding, the beads were washed and incubated with 0.1–0.5 μg of TEV protease in 4 mL 1X TBS for 8 hours. The resulting flowthrough was incubated with equilibrated CaM-sepharose beads (GE Healthcare) in the presence of 2 mM CaCl₂ for 12 hours. Finally, 0.5mL elutions were obtained using 1X TBS with 4 mM EGTA. In the case of some proteins, only the CaM-sepharose bead binding was performed, and eluted as above. Elutions were pooled and concentrated via 10K, 30K, or 100K Amicon Ultra centrifugal filtration devices (Millipore) as necessary.

Boiling experiment

Purified protein in 50 mM Tris pH 7.5, 100 mM NaCl, 4 mM EGTA was concentrated via Amicon Ultra centrifugal filtration devices (Millipore) or diluted to 0.5 μM, and 200 μL was boiled at 95°C or placed on ice (4°C) for 10 minutes. Samples were then centrifuged at 20817 g for 5 minutes at 4°C, washed twice with buffer, and resuspended in protein sample

buffer. Supernatants and pellets were run on an SDS-PAGE gel and stained with Gel Code Blue (Pierce).

Limited Proteolysis

Purified protein at a concentration of 0.5 μM was incubated with 0.1 μM chymotrypsin in a total volume of 50 μL . Protein sample buffer was added after a 5 minute incubation on ice. Samples were run on a 7.5–20% SDS-PAGE gel and stained with Gel Code Blue (Pierce).

Circular dichroism (CD)

CD spectra were obtained on an AVIV 62DS Circular Dichroism spectropolarimeter. For optimal spectra, proteins were purified in 10 mM Tris-HCl pH 7.5, 10 mM NaF at a concentration of 50–100 μM as determined via Bradford. Wavelength scans were performed at 4°C in a 1 mm path length cuvette using a 1 nm step size, a 3 s averaging time, and 5 repeats.

Size-exclusion chromatography

Chromatography was performed using a Superose 6 gel filtration column (GE Healthcare). The following proteins were used to determine the apparent molecular weight: thyroglobulin, aldolase, ferritin, carbonic anhydrase, ovalbumin, and ribonuclease A. Protein was purified from yeast in 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 4 mM EGTA, and 800 μL at a concentration of 1 μM was added to the column after the column was equilibrated with buffer. Once loaded, 20mL of buffer was added to the column and collected in 0.5 mL increments over 30 mL. Samples were precipitated with 10% trichloroacetic acid, centrifuged at 20817 g for 5 minutes at 4°C, washed once with cold acetone, and resuspended in 5% of the original volume. The samples were then run on an SDS-PAGE gel and stained using Gel Code Blue (Pierce).

Velocity sedimentation

Sedimentation via centrifugation was performed using a 5 mL 1–10% sucrose gradient poured using a Labconco gradient maker in Beckman ultracentrifuge tubes. Protein was purified as above, and 100 μL of 0.5–1 μM purified protein was added to the gradient and centrifuged at 55000 g for 17.5 hours at 4°C in a Beckman ultracentrifuge using a swinging bucket rotor (SwTi55). Elutions of 0.5 mL were collected, TCA precipitated as above, and resuspended in 5% of the original sample volume. The samples were run on an SDS-PAGE gel and stained using Gel Code Blue (Pierce).

Calculation of molecular weight from Stokes radius and Svedberg coefficient

Molecular weight was determined using the gel filtration and velocity sedimentation data in accordance with the Siegel and Monty equation:

$$M_r = \frac{6\pi\eta NR S}{1 - v\rho} \quad [1]$$

where M_r is the apparent molecular weight, η is the viscosity of the medium, N is Avagadro's number, R is the Stokes radius calculated from gel filtration experiments, S is the Svedberg coefficient calculated from velocity sedimentation experiments, v is the partial specific volume, and ρ is the density of the medium¹⁵. The partial specific volume of the full protein was calculated given the v of each amino acid¹⁶. Standard values for the density of water ($\rho = 1 \text{ g/cm}^3$) and buffer viscosity ($\eta = 0.01005 \text{ g/cm}_x\text{s}$) were used. Molecular weight values based on primary amino acid sequence alone were calculated using the Protein Extinction Coefficient Calculator (www.biomol.net/en/tools/proteinextinction.htm).

Differences between calculated and apparent molecular weight revealed the oligomeric state of the protein.

Affinity pulldown experiments

Recombinant proteins containing GST- and -TAP tags were induced in yeast as described above. Yeast powder (2 g) containing the GST-tagged proteins (see above) were solubilized in pulldown buffer (0.1 M sorbitol, 20 mM HEPES, 30 mM potassium acetate, and 2 mM EDTA pH 7.8), and 100 μ L protease inhibitor cocktail for fungal and yeast extract (Sigma). The lysate was then dialyzed overnight, using Spectra/Por Dialysis Membrane (MWCO: 6–8,000, Spectrum Labs) in pulldown buffer to dilute the endogenous yeast glutathione (1L at 4°C for 2 hours, repeated overnight). After dialysis, the lysate was incubated with 100 μ L of a 50% slurry of pre-equilibrated glutathione-agarose beads (Sigma) in the presence of 3 mM DTT for 2 hours rotating at 4°C. The beads containing the GST-tagged protein were then washed 2 times with pulldown buffer + 0.2% Tween-20 and run on an SDS-PAGE gel to determine equivalent protein amounts to perform the pulldown.

Pan1-TAP constructs were purified using 1X TBS buffer in a similar manner as detailed in *Yeast protein purification*, exchanged into pulldown buffer using a P-10 desalting column (GE Healthcare), and 80 μ L at a concentration of 10 μ M was added to ~20 μ L glutathione-agarose beads containing GST-tagged constructs, incubated for 45 minutes at 4°C, washed as above, and resuspended in 15 μ L protein sample buffer with 2 mM ME (PSB). The supernatant was added to 4X PSB and both the supernatant and pellet were run on a 7.5–15% SDS-PAGE gel. The gels were then either stained with GelCode Blue (Pierce, Rockford, IL) or transferred to nitrocellulose by electrophoresis, blocked with 5% nonfat dry milk in Tris buffer, and incubated with horseradish peroxidase (HRP)-conjugated Donkey anti-Rat antibodies (1:5000, Pierce) that recognize the IgG-binding component of the TAP-tag. The blot was then washed and developed using chemiluminescence reagents, and imaged using an Alpha Innotech Fluorochem. Blots were then reprobed using an anti-GST antibody (1:5000, Invitrogen), washed and incubated with Goat anti-Mouse HRP antibodies (1:5000, Pierce), and re-imaged as above.

Tryptophan fluorescence and quenching experiments

Fluorescence was monitored using a T-format PTI fluorimeter (Monmouth Junction, NJ) with 75W xenon arc lamp. Protein in 1X TBS and 4mM EGTA was placed in a quartz cuvette with a 1.5 cm path length. The sample was excited with light at a wavelength of 295 nm at 20°C and the emission spectrum from 310 nm to 410 nm was obtained at 1 nm intervals. For quenching experiments, stock solutions of 5 M acrylamide, potassium iodide, or cesium chloride were prepared by dissolving each in 1X TBS and 4 mM EGTA. The quencher was added in increments of 0.03 M and a full emission spectrum was obtained at each concentration of quencher. The spectra were corrected for both the increase in volume and the emission spectra using buffer alone to remove Raman scattering. For acrylamide quenching, all intensities were corrected for acrylamide absorption at 295nm using the equation:

$$F_{corr.} = 10^{\left(\frac{l\epsilon[acryl]}{2}\right)} F_{meas.} \quad [2]$$

where $l=1.0$ cm is the cell length and an extinction coefficient of 0.23 L/(mol $\times$ cm) was used for acrylamide, as previously reported^{17,18}. Using the initial spectra maximum, titrating in defined concentrations of quencher, and measuring the intensity of the emission at λ_{max} , a Stern-Volmer constant (K_{SV}) was determined using the slope given the following equation:

$$F_0/F = 1 + K_{SV} [Q] \quad [3]$$

where F_0 is the initial emission maximum, F is the emission maxima given the concentration of quencher, $[Q]$. For non-linear Stern-Volmer plots, K_{SV} were taken for the initial slope of the line.

Time-resolved fluorescence experiments

Time-resolved fluorescence intensity and anisotropy measurements were made using a fluorometer built for this purpose, and data analysis was performed as previously described¹⁹. Briefly, frequency-doubled output from a cavity-dumped dye laser synchronously pumped by a mode-locked YAG:Nd laser was used to excite fluorescence; the exciting radiation at 300 nm was polarized vertically and comprised a train of 15 ps pulses separated by 245 ns intervals. Fluorescence emission was registered by two wings in a T-format. In each wing the radiation first passed through a polarizer set at either 0° or 90°, a lens, a depolarizer, a monochromator tuned to 345 nm central wavelength (16 nm bandwidth), and was detected by a microchannel plate photomultiplier R1564U. Time-correlated single-photon counting (TCSPC) electronics sorted the anode pulses from the photomultipliers by their time delays relative to the exciting pulse; the resulting photon counts were stored in 2048 channels (13.333 ps/channel) of a multichannel analyzer (MCA). A fluorescence measurement with one protein lasted about 80 min. and consisted of 40 repetitions of a 120 s cycle. During each cycle each wing measured vertically polarized emission for 50 s, horizontally polarized emission for 50 s, and the impulse response function (IRF) for 20 s. While one wing measured the vertical polarization (0°), the other wing always measured the horizontal polarization (90°) and vice versa. The IRF was measured using a ludox scatterer cell with the polarizers set to 45° and the monochromators tuned to 300 nm. The IRF recorded using the scatterer cell was about 5 channels or 65 ps fwhm, representing the overall time resolution.

For analysis of the time-resolved anisotropy data, vertical and horizontal polarization fluorescence were fit by a model using the following system of equations:

$$I_v(t) = g^{+1/2} [1 + 2r(t)] I(t) \quad [4]$$

$$I_h(t) = g^{-1/2} [1 - r(t)] I(t) \quad [5]$$

$$r(t) = \sum_{m=1}^{N_{ani}} \beta_m \exp\left(\frac{-t}{\tau_m}\right) \quad [6]$$

$$I(t) = \sum_{n=1}^{N_{int}} \alpha_n \exp\left(\frac{-t}{\tau_n}\right) \quad [7]$$

Numerical convolutions of I_v and I_h with the IRF were simultaneously fit to the vertical and horizontal fluorescence data. The amplitudes α_n and the time constants τ_n in equation [7] describe the behavior of the total emission intensity $I(t)$. In the case of a monoexponential $I(t)$, $N_{int}=1$, τ_1 represents the excited-state lifetime. Likewise, the amplitudes β_m and the time constants τ_m in equation [6] describe the behavior of the time-resolved anisotropy $r(t)$. In the case of a monoexponential $r(t)$, $N_{ani}=1$, τ_1 represents the rotational correlation time.

The anisotropy $r(t)$ depends only on the rotation of the Trp sidechain. The total emission intensity $I(t)$ depends on the radiative and non-radiative decay mechanisms, but is independent of the rotation of the Trp sidechain. Thus, the model parameters τ_m and τ_n describe to the rotational dynamics of the Trp sidechain, and the model parameters ϕ_m and ϕ_n describe the radiative and non-radiative decay mechanisms. The values of the model parameters τ_m , τ_n , ϕ_m , and ϕ_n were optimized to minimize the χ^2 , which was defined as the sum over all MCA channels (including both vertical and horizontal polarization) of the weighted squared differences between the TCSPC data and the numerical convolution of the model function in equations [4–7] with the IRF. A previously described global nonlinear least-square program polartcp¹⁹ was used to find the best values of the fitting parameters τ_m , τ_n , ϕ_m , and ϕ_n . The ratio of the instrument sensitivity to vertical and horizontal polarization was represented by the parameter g , which was not treated as a free fitting parameter in this study, since its value was independently determined with the accuracy of $\pm 0.1\%$ or better. The instrument was equipped with achromatic depolarizers DPU-15 (Optics for Research, Caldwell, NJ) that were placed between the polarizers and the emission monochromators in each of the two emission wings. It was found that these depolarizers cancelled the polarization dependence of the instrument's sensitivity with a great accuracy (better than 0.1%), therefore a non-unit g -factor could arise only from unequal excitation intensities or unequal photon counting times for the vertical and horizontal polarization. To cancel the effects of slow drifts in the laser intensity on the g -factor, each emission wing was alternatively used to count vertical and horizontal polarization photons, furthermore, while the left wing was counting vertically-polarized photons, the right wing was counting horizontally-polarized photons, and vice versa. The vertically- and horizontally-polarized photons were counted for equal real times, however, due to the dead time of the multichannel analyzers, the live time was always slightly shorter for the vertical polarization, which had a higher intensity, and, therefore, a higher dead time. The multichannel analyzers (EG&G Ortec ADCAM 918) keep track of the real time, the dead time, and the live time, which equals the real time minus the dead time. The g -factor was calculated as the ratio of the live time for the vertical polarization to the live time for the horizontal polarization.

The same set of TCSPC data was analyzed multiple times using different combinations of N_{int} and N_{ani} , where N_{int} is the total number of exponentials in $I(t)$, and N_{ani} is the total number of exponentials in $r(t)$. Increasing either N_{int} or N_{ani} from 1 to 2, then to 3, etc., resulted in a significant initial decrease in the reduced χ^2 , which stopped abruptly when the number of exponentials exceeded a certain level. Of all the combinations of N_{int} and N_{ani} that resulted in the smallest (and essentially the same) reduced χ^2 value, we chose the combination with the smallest N_{ani} and the smallest N_{int} ; equations [4–7] with these values of N_{int} and N_{ani} define the best model function. The quality of the fits for the best model function were also examined using plots of weighted residuals and plots of the autocorrelation of unweighted residuals. The values of the fitting parameters τ_m , τ_n , ϕ_m , and ϕ_n corresponding to the best model function are shown in the results section (instead of τ_n we give $\% \tau_n$, which equals τ_n divided by the sum of all τ_i). The alpha-weighted mean lifetime was calculated by taking the sum from $n=1$ to N_{int} of the products $\tau_n \times \% \tau_n$; the values τ_n and $\% \tau_n$ were taken from Table II, and the resulting alpha-weighted mean lifetime is shown in the third column of Table I.

RESULTS

Pan1 has multiple regions of predicted intrinsic disorder

Pan1 is a large, essential protein with many binding partners, many of which are involved in endocytosis^{9,20,21}. While no regions of Pan1 have had their structure solved, prediction programs show that the majority of the protein is random coil (60.58%), with a lesser

amount of alpha-helices (23.26%) and extended beta-sheets (5.25%) (data obtained from averaging eight secondary structure prediction programs using NPS@: network protein sequence analysis²²) (Figure 1A). This is similar to a number of other endocytic proteins, where tandem, folded domains are punctuated by flexible peptides of random coil²³. To further probe the importance of Pan1 structure and its function, we used an algorithm that predicts naturally disordered regions (PONDR) in a given amino acid sequence, based on a neural network of known ordered and disordered regions^{24–26} (Figure 1B). The Pan1 protein has two major domains of expected order, its EH domains, spanning amino acids 263–358 and 593–688. The large stretches of predicted disorder occur at both the N- and C-termini of Pan1, consisting of the N-terminal region between the EH domains, which is known to be highly phosphorylated^{21,27}, and its C-terminal proline-rich domain, which is characteristically known to be mostly disordered in other proteins^{24,25}. The central region, while predicted to be disordered, has four short stretches and one extended high-probability stretch of predicted coiled-coil domains as determined using the COILS server²⁸ (amino acids 687–700, 790–805, 905–918, 1020–1043 – all shown in light green; and amino acids 1131–1190, corresponding to the extended coiled-coil domain – shown in green in Figure 1A).

To validate the PONDR predictions, we assayed for the presence of folded vs. unstructured domains using a boiling and pelleting experiment (Figure 2A). In general, disordered regions do not pellet after heating while structured regions tend to unfold upon boiling and aggregate upon cooling, causing them to be found in the pellet after centrifugation²⁹. Pan1 N-terminal, central, and C-terminal fragments were boiled, centrifuged, and the supernatant and pellet fractions resolved by SDS-PAGE. The N-terminal fragment (His₆-Pan1₂₃₁₋₇₀₀) pelleted after boiling, as expected for a protein with folded regions (Figure 2A). In contrast, the C-terminal fragment (His₆-Pan1₁₁₉₁₋₁₄₈₀) did not pellet after boiling, consistent with the unstructured prediction from the PONDR analysis. To complete the analysis, the central region of Pan1 was also examined. Pan1⁶⁸⁹⁻¹¹⁹⁰ was purified from yeast using a TAP-tag (see Methods); this fragment cannot be expressed in bacteria. Only a small fraction of the protein was found in the pellet after boiling; a similar amount was found in the pellet for the control condition, ten minutes on ice, possibly due to self-assembly, as this region has been implicated in self-association³⁰.

To further examine each of these Pan1 regions and reveal possible stable domains, limited proteolysis was performed using chymotrypsin (Figure 2B). After incubation on ice with 0.1 M chymotrypsin for 5 minutes, each of the purified proteins showed significant degradation. The second EH domain alone was somewhat subject to proteolysis by chymotrypsin (Figure 2B, lanes 2 and 3), indicating that the folded domain still has proteolytically accessible sites. When protease was added to Pan1 N-terminus (amino acids 231–700), a pair of bands around 21 kDa, possibly representing each EH domain, was revealed (Figure 2B, lanes 4 and 5). The central domain appeared to be very proteolytically sensitive, as very few bands were observed following protease incubation (Figure 2B, lanes 6 and 7). Self-interaction, or possibly aggregation, of the central domain can be observed at around 200 kDa. Although these samples were subjected to reducing agents, this 200 kDa band persisted; similar stable oligomers in the presence of reducing agents have been found previously for synaptophysin³¹. Finally, the C-terminus had a proteolytically stable region at approximately 32 kDa, presumably due in part to a span of sequence without chymotrypsin cut sites (amino acids 1290–1423) (Figure 2B, lanes 8 and 9). This proline-rich portion of Pan1 is known to migrate at a larger molecular mass than predicted³².

Circular dichroism (CD) measurements yielded results that were consistent with the boiling and pelleting experiment for the N- and C- termini (Figure 2C). Pan1 N- and C-terminal fragments were purified from bacteria as described in the Methods section. The Pan1 N-

terminal fragment (amino acids 230–700) exhibited the typical CD spectrum for α -helices, with peaks at 222 and 208 nm, while the CD spectrum of the C-terminus (amino acids 1191–1480) indicated that this region is mostly random coil.

The central region of Pan1 behaves as a high molecular mass species

We further analyzed the central region of Pan1, which is predicted to contain periodic, short coiled-coil domains while being otherwise unstructured (COILS server,²⁸). We have previously characterized the formation of Pan1 homodimers via this region of the protein³³, but the specific region that mediates this interaction had not been characterized. To begin our analysis of this region of Pan1 (aa689–1190), we used gel filtration and sucrose gradient velocity sedimentation to calculate a Stokes radius of 74 Å and a Svedberg coefficient of 4.5S, respectively (Figure 3A,B). These values were determined using reference standards for each experiment. Using the Siegel and Monty equation with the observed values¹⁵, we calculated a molecular mass of 139 kDa for the Pan1⁶⁸⁹⁻¹¹⁹¹ fragment. This value was nearly twice the predicted value of 76.9 kDa obtained using a calculated molecular mass based on primary sequence (see Methods), consistent with this region of Pan1 forming a homodimer. These biochemical data confirmed previously obtained yeast two-hybrid data consistent with the formation of Pan1 homodimers (see Table 2 from reference³⁰). Interestingly, the yeast two-hybrid data showed that increasing the length of the C-terminus appeared to inhibit the homotypic interaction between two Pan1 central-region fragments. This may be due to a C-terminal obstruction of the region that mediates dimer formation. We next aimed to test the yeast two-hybrid generated hypothesis through affinity pulldown experiments.

Pan1 amino acids 705-848 are critical for homotypic interaction

To map the specific region of Pan1 necessary for homodimer formation, we generated Pan1 fragments with either TAP- or GST-tags for expression and purification from yeast. We conducted pulldown experiments of purified Pan1-TAP fragments with GST-Pan1 fragments coupled to glutathione beads (Figure 4A). Glutathione beads bound with GST-tagged Pan1 containing pieces of the central region (Pan1^{543-948/1432-1480}, Pan1⁵⁴³⁻⁷⁰⁰, Pan1⁵⁴³⁻⁸⁵⁰, Pan1⁹⁴⁸⁻¹⁴⁸⁰) or GST alone were incubated with TAP-tagged Pan1 fragments of varying length (Figure 4B). Nearly full-length Pan1 (Pan1²³¹⁻¹⁴⁸⁰-TAP) bound GST-Pan1^{543-948/1432-1480} and GST-Pan1⁵⁴³⁻⁸⁵⁰, but did not bind GST-Pan1⁵⁴³⁻⁷⁰⁰ or GST-Pan1⁹⁴⁸⁻¹⁴⁸⁰ more than GST alone, implicating the region of 700–850 as important for self-interaction. Differences between binding efficiency can be observed when comparing the ability of the Pan1 GST-tagged constructs to pulldown Pan1²³¹⁻¹⁴⁸⁰-TAP or the central region of Pan1, Pan1⁶⁸⁹⁻¹¹⁹⁰-TAP. Notably, the presence of the regions flanking the central domain of Pan1²³¹⁻¹⁴⁸⁰-TAP seemed to inhibit the central domain interaction with GST-Pan1^{543-948/1432-1480} and GST-Pan1⁵⁴³⁻⁸⁵⁰. This confirmed the yeast two-hybrid data and possibly implicates the C-terminus of Pan1 in the disruption of this interaction. To further refine the interaction interface, additional Pan1 C-terminal TAP-tagged constructs were generated with increasing amounts of the central region of Pan1. The Pan1-TAP fragment containing amino acids 705-1480 was able to bind the GST-Pan1 construct but the fragments containing amino acids 848-1480 and 990-1480 had weak or no binding. Together, these results implicate amino acids 705-848 as critical for a Pan1-Pan1 self-interaction that may be regulated through an interaction with its C-terminus.

Pan1 emission spectra reveal solvent-accessible tryptophans

We next probed the structure and dynamics of Pan1 using tryptophan fluorescence. The Pan1 protein has four endogenous tryptophans, each in a functionally significant region of the protein (Figure 5A). The first two tryptophans (Trp³¹² and Trp⁶⁴²) are in the ligand-binding pocket of each of the two Eps15 Homology (EH) domains. These tryptophans are

essential for the well-characterized interaction of an EH domain with asparagine-proline-phenylalanine tripeptide motif ligands. While the second EH domain of Pan1 has an affinity for this ligand, the first EH domain does not³⁴. The third tryptophan (Trp⁹⁵⁷) is in the central region of Pan1, a region containing predicted coiled-coil domains. Finally, the fourth tryptophan (Trp¹²⁸⁰) is in the C-terminus of the protein, and has been shown to be an important residue in the motif involved in Arp2/3 activation¹¹. The importance of this tryptophan has been tested in other proteins as well, and has been found to be an essential residue for Arp2/3 activation in cortactin³⁵.

Tryptophan fluorescence experiments require large amounts of purified soluble protein, which were produced as C-terminally TAP-tagged Pan1 from the over-expressing plasmid pEG-KT (Figure 5B)¹⁴. We found that full-length Pan1 protein formed insoluble aggregates; this problem was resolved by removing the first 230 amino acids. Amino acids 1–230 of Pan1 are glutamine and asparagine rich, and cause the protein to form amyloid fibrils when purified at high concentration (Giancarlo Costaguta and Gregory Payne, *pers. comm.*; B. Daniel Pierce and Beverly Wendland, *unpub. observations*). Pan1 protein lacking the first 230 amino acids is able to complement a *pan1* strain grown at 30° or 37°C (see Figure 6B and data not shown), suggesting that the first 230 amino acids are dispensable for major Pan1 functions. The experiments below were all performed with Pan1^{231–1480} protein unless noted otherwise. To remove the contribution of the tryptophan in the TAP-tag, the tryptophan in the calmodulin binding-peptide was mutated to phenylalanine using site-directed mutagenesis, which did not affect the binding of the TAP-tag to CaM beads. After sequential purification on IgG- and CaM-sepharose, we first analyzed the wild-type Pan1^{231–1480} protein by obtaining the emission spectrum of the protein excited at 295 nm (Figure S1). The results showed that the four tryptophans were moderately solvent accessible, as the peak of the spectrum is blue shifted to 342 nm, by comparison to the 352 nm peak obtained for a control solution of free L-tryptophan.

To further determine the degree of tryptophan accessibility, we performed a series of quenching assays. Acrylamide is a small, neutral molecule that is commonly used to quench the fluorescence of tryptophan in the context of its polypeptide chain. When compared with L-tryptophan in solution, the Pan1 tryptophans were much less accessible (Figure S2A). This conclusion derives from the Stern-Volmer plot used to calculate the K_{SV} , where the original intensity maximum is taken as a ratio to the intensity maximum of the spectrum when quencher is added and plotted over the concentration of quencher (see Methods). The K_{SV} of Pan1 when quenched with acrylamide is 4.8, which indicates moderate solvent accessibility³⁶, by comparison to a measured K_{SV} for L-tryptophan alone of 20.3 (Figure S2A), similar to a previously reported value of 20.0 ± 2 ¹⁸. Thus, the acrylamide quenching of Pan1 tryptophans confirms the blue shift seen in the emission maximum when excited at 295 nm. While the slope of a Stern-Volmer plot represents a measure of solvent accessibility, the upward curvature of this plot serves as an indication that both collisional and static quenching mechanisms have significant contributions to the data³⁶. Collisional quenching gives a quantifiable value for how often the quencher comes in contact with the tryptophan residue based on its accessibility. Unlike collisional quenching, static quenching is a result of non-covalent chemical interaction between the fluorophore and the quencher, where the resulting complex is not fluorescent. The combination of the collisional quenching with static quenching explains the upward slope seen in acrylamide quenching of L-tryptophan, as the concentration of acrylamide in the solution increases. That the plot of Pan1 is linear, however, indicates that only one quenching mechanism is present, which has to be the collisional mechanism, the most common quenching mechanism in the case of acrylamide and most other quenchers. The static quenching is probably prevented by some degree of burial of the Pan1 tryptophan residues.

As an additional approach to observe Pan1 tryptophan environments, potassium iodide quenching was performed. While both iodide and acrylamide are commonly used quenchers, iodide is more subject to electrostatic interactions with any positively charged amino acids that may be near the tryptophan being studied³⁷. When Pan1 was quenched using potassium iodide, we obtained a Stern-Volmer constant of 4.0, which is less than the K_{SV} of tryptophan alone, 15.0 (Figure S2B). When compared with reported K_{SV} values whose extremes can range from effectively 0 to 13.0³⁶, these data indicated that the K_{SV} of Pan1 is in the intermediate range, containing slightly buried tryptophans that are also accessible to collisional quenchers.

To learn more about conformational dynamics of the different regions of Pan1, we assessed the specific accessibility of each of the Pan1 tryptophans individually. Four single-tryptophan mutant proteins (hereby referred to as W312, W642, W957, and W1280) were generated by mutating three of the four tryptophans to phenylalanine. Each of these proteins expressed well, was soluble, and complemented a *pan1* strain grown at both 30 and 37°C, indicating that the mutant proteins are functional (Figure 6A,B). Additionally, each of these mutants appeared to exhibit roughly the same size and shape, as indicated by velocity sedimentation experiments (Figure 6C). When excited at 295 nm, each of the four tryptophan mutants showed varying emission spectra distinct from the wild-type protein (Figure 7A). Several observations about the contributions of each of the Pan1 tryptophans could be derived from these data. First, the majority of the fluorescence intensity seen in the wild-type protein seemed to be generated by the second tryptophan, Trp⁶⁴², as the W642 mutant has an intensity closest to wild-type levels. The remaining tryptophans may be in environments that naturally quench tryptophan fluorescence. Normalization of these emission spectra allowed us to compare the spectra more closely (Figure 7B). Notably, the first and third tryptophans, Trp³¹² and Trp⁹⁵⁷, appeared to be shifted toward shorter wavelengths when compared to wild-type protein, indicating that these are slightly more buried than the other two tryptophans. The second and fourth tryptophans, on the other hand, appeared to be shifted toward longer wavelengths. Acrylamide quenching of each of these mutant proteins confirmed the information garnered from the emission spectra themselves (Table I and Figure 8A). Each of the Pan1 tryptophans was moderately accessible to quenching by the neutral, polar acrylamide molecule, with Trp¹²⁸⁰ as the most solvent-exposed of the four.

To further develop our understanding of the Pan1 molecule from these initial experiments, the alpha-weighted mean lifetimes of each single tryptophan mutant were determined (Table I). From the fluorescence lifetime, a biomolecular quenching constant could be determined, as governed by these equations:

$$\frac{F_0}{F} = 1 + K_{SV} [Q] = \frac{\tau_0}{\tau} \quad [8]$$

$$= 1 + k_q \tau_0 [Q] \quad [9]$$

where τ_0 is the initial fluorescence lifetime, τ is the lifetime in the presence of the quencher Q , K_{SV} is the Stern-Volmer constant and k_q is the bimolecular rate constant. The bimolecular rate constants were determined for each quencher and each mutant (Table I). However, these values are only particularly useful for proteins undergoing both static and collisional quenching; the lack of an upward curvature seen in the acrylamide quenching of each tryptophan mutant indicates a dominant contribution of collisional quenching over static quenching, especially when compared with tryptophan alone (Figure S2, Figure 8A, and ³⁸). For proteins that undergo static quenching, the rate constant can give a sense of the

rate at which a tryptophan becomes accessible to its environment due to fluctuations of the peptide backbone. In the case of Pan1, the W642 protein had a lifetime approaching twice the values as the other three single-tryptophan mutants, yielding a k_q value for acrylamide quenching approximating half that of the other mutants. Due to the lack of static quenching in the presence of acrylamide, this difference was most likely solely a reflection of the higher fluorescence lifetime of Trp⁶⁴² and not indicative of the tryptophan accessibility.

The first and fourth Pan1 tryptophans are in opposite electrostatic environments

Unlike the acrylamide quenching data, which showed that each tryptophan mutant had a similar K_{SV} value, potassium iodide quenching experiments revealed differences between the K_{SV} values for the tryptophan mutants. The most N-terminal tryptophan, Trp³¹², located in the binding pocket of the first EH domain, appeared to be highly quenched by iodide, as evidenced by a Stern-Volmer constant of 6.7 (Table I and Figure 8B). In direct contrast, the tryptophan located in the second EH domain, Trp⁶⁴², was not subject to potassium iodide quenching to the same degree, with a K_{SV} of 2.0. The tryptophan in the central region of the protein (Trp⁹⁵⁷) had a K_{SV} of 2.6 and the fourth tryptophan (Trp¹²⁸⁰), in the Arp2/3 activation site of Pan1, had a K_{SV} of 2.3, both indicative of an intermediate level of quenching by potassium iodide.

To resolve the inconsistencies between the acrylamide and potassium iodide quenching experiments, the alternative quencher cesium chloride was used. The cesium cation has the opposite charge of the iodide anion, distinguishing between quenching due to an electrostatic effect versus solvent accessibility³⁹. Cesium is a less effective quencher than either acrylamide or iodide, as we observed a lower Stern-Volmer constant for a solution of tryptophan alone (K_{SV} = 20.3 for acrylamide, K_{SV} = 15.0 for KI, and K_{SV} = 1.9 for CsCl) (Figure S2A–C). Cesium chloride quenching of the tryptophan mutant proteins was similar to that of wild-type protein, except that W1280 was highly quenched (K_{SV} = 1.9). In contrast to the W312 protein, this result is almost assuredly due to the large number of negatively charged amino acids surrounding Trp¹²⁸⁰ that contribute to an electrostatic effect. The sequence surrounding the fourth tryptophan is <DEDEDDGWSDEDE>, where ten negatively charged amino acids are within seven amino acid positions upstream or downstream of the tryptophan. By contrast, the W312 protein showed almost no quenching in the presence of cesium chloride (Figure 8C). Threading the first EH domain of Pan1 onto the known structure of the second EH domain of mammalian Eps15 (PDB: 1EH2), predicts that positively-charged amino acids Arg²⁹⁵, Arg³¹⁰, and Lys³¹⁹ are adjacent to Trp³¹² (B.D.P. and B.W.; unpublished results). This prediction and the quenching data support the hypothesis that it is the electrostatic environment around Trp³¹² that contributes to its high degree of quenching in the presence of potassium iodide.

Self-interactions create unique environments for Pan1 regions

To further examine Pan1 protein conformations using the tryptophan mutants, we performed time-resolved fluorescence anisotropy measurements. Time-resolved anisotropy $r(t)$ contains information about the conformational dynamics in the local environment of the intrinsically fluorescence tryptophan residue (at $t < 0.5$ ns), as well as about the rotational diffusion rate of the structural domain that contains the tryptophan (at $t > 5$ ns). The rotational diffusion rate serves as a reflection of the domain size. Time-resolved anisotropy experiments were performed as described previously¹⁹ for each of the Pan1^{231–1480} single tryptophan mutants, to determine the local flexibility in the local environment of each tryptophan residue dependent on the rigidity of the protein region. Additionally, these types of data can generate a rough molecular mass estimate of the region containing the tryptophan.

Vertically and horizontally polarized time-resolved fluorescence data were analyzed simultaneously (see Methods), which yielded the model parameters describing the time-resolved intensity and anisotropy. The time-resolved intensity, $I(t)$, contains five exponential terms (Table II). The time-resolved fluorescence anisotropy, $r(t)$, contains three exponential terms (Table III). Each $r(t)$ curve was then reconstructed by substituting the parameters β_m and τ_m from Table III in the equation:

$$r(t) = \sum_{m=1}^3 \beta_m \exp\left(\frac{-t}{\tau_m}\right) \quad [10]$$

where $r(t)$ is the time resolved anisotropy, t is time, and β_m and τ_m are the amplitude and the time constant for the exponential term number m , respectively. The $r(t)$ curves reconstructed in this way are shown in Figure 11; these curves are characteristic of Trp residues in tightly-packed protein regions, which prevent the Trp sidechain from spinning freely on the time scale between 0.1 ns and 0.25 ns. The protein as a whole has flexibility, since $r(t)$ is not monoexponential.

For W312 and W642 the time constant τ_1 was very short and close to the time resolution limit of the instrument. The exponential terms with very short τ_1 can be attributed to the contamination of tryptophan fluorescence emission by the scattered light that is particularly strong due to the high molecular mass of Pan1. The systematic errors apparent in the plots of weighted residuals and autocorrelations of residuals in Figures 9 and 10 were also consistent with the scattered light contamination. In contrast, the value of τ_1 for W957 and W1289 was considerably longer than the time resolution limit and therefore cannot be attributed to scattered light; this τ_1 is consistent with the rotation of a very small protein segment, possibly, the rotation of tryptophan sidechain about the C α -C and C-C bonds in an unstructured local environment (in water this rotation would be considerably faster). The values of $\tau_1 = 0.05$ and $\tau_1 = 0.35$ ns obtained with the W1280 mutant were consistent with fast conformational dynamics (fluidity) in the environment of Trp¹²⁸⁰ and this usually indicates a locally unstructured region. In the case of the W957 mutant the amplitude $\beta_1 = 0.03$ was smaller, and the time constant $\tau_1 = 0.53$ ns was longer, indicating a slower conformational dynamics (less fluidity) in the environment of Trp⁹⁵⁷. The environments of Trp⁶⁴² and Trp³¹² appeared to be rigid (no fluidity).

The second exponential for each mutant represents the restricted rotation of a protein segment, which can be also thought of as bending of the protein. Based on the values of τ_2 , which fall between 2 and 4 ns, the size of the rotating segment was estimated to be between 4 and 8 kDa. The rotation/bending angle is small for all four tryptophans, judging by the small amplitude (β_2) of this term.

Finally, the third exponential in $r(t)$ can be used to estimate the molecular mass of the protein domain containing the tryptophan. Based on τ_3 values, the Trp³¹² appeared to be in a freely rotating protein region of 44 kDa, 83 kDa for the Trp⁶⁴², 140 kDa for the Trp⁹⁵⁷, and 239 kDa for Trp¹²⁸⁰. The estimations were made assuming that all protein domains are rigid and spherical, contain no internal water pockets, and are connected very loosely (by very long and completely flexible linkers). These assumptions are almost certainly not correct given the predictions of secondary structure and disorder (see Figure 1A,B), but can still provide a rough sense of the relative mass of the regions that contain each tryptophan.

A plot of the time-resolved fluorescence anisotropy is shown in Figure 11. Comparing the slow rotation of the Trp-containing domains in the first two Pan1²³¹⁻¹⁴⁸⁰ mutants, W312 and W642, which is reflected by the tails of $r(t)$ curves at $t > 5$ ns, we found that the anisotropy of the tryptophan in the W312 protein decreased about twice as fast as the

anisotropy of the tryptophan in the W642 protein (Figure 11). These two proteins as a whole did not appear to be grossly different in their molecular weight based on velocity sedimentation (see Figure 6C), leading to the interpretation that Pan1 is structured such that at least the two rigid domains containing Trp³¹² and Trp⁶⁴² are connected by a flexible linker and can rotate independently, with the domain containing Trp⁶⁴² having a rotation that is twice slower than the domain containing Trp³¹². Trp³¹² and Trp⁶⁴² are each in EH domains that are expected to have similar structures and similar molecular masses. The slower rotation of the EH domain containing Trp⁶⁴² may be qualitatively explained by the fact that this domain is attached to two anchors (the first EH domain and the central dimerization domain), while the EH domain containing Trp³¹² is attached to just one anchor (the second EH domain). Additionally, the first 230 amino acids of Pan1 that are absent in our samples may have properties that change the degree of flexibility or folding of Trp³¹².

Trp⁹⁵⁷ and Trp¹²⁸⁰ appeared to be in larger domains, 140 kDa for Trp⁹⁵⁷ and 239 kDa for the Trp¹²⁸⁰. These values were possibly overestimated due to the assumption that these tryptophans are located within spherical bodies. Both of these tryptophans are in regions that predict local order in a larger region of disorder (see Figure 1B). Our other data indicated that Pan1 exists as a dimer, mediated by interactions of the central region, and this dimer may explain why the anisotropy data implicated larger molecular mass domains present in Pan1. Surprisingly, W1280 had an anisotropy consistent with a very high molecular mass species, implicating Trp¹²⁸⁰ as engaged in additional oligomer formation or potential intramolecular interactions that could increase the apparent molecular mass of this region.

DISCUSSION

The work presented here characterizes the Pan1 protein as at least a dimer consisting of multiple domains separated by flexible linkers. *PAN1* is an essential gene in *Saccharomyces cerevisiae*, coding for an endocytic scaffold with many binding partners. Due to its high degree of protease-sensitivity, the bacterial toxicity when expressing its central region, and its propensity to form amyloid fibrils, full-length Pan1 protein has not been subjected to many biochemical or biophysical studies. In this study we provide an analysis of Pan1 conformation and develop a basis for future studies of through the characterization of its multiple domains.

The work described here provides the groundwork for the designation of Pan1 as an intrinsically disordered protein. Secondary structure predictions correlate well with the PONDR predictions of intrinsic disorder; that is, several random coil regions of Pan1 should be formally classified as intrinsically disordered regions (IDR) and placed in the DisProt database of disordered proteins⁴⁰. Many other mammalian endocytic proteins have large regions of random coil that implicate them as containing IDRs, and of the 28 proteins analyzed in one study, only five mammalian endocytic proteins had a higher percentage of predicted random coil regions than Pan1 (60.56%): Dab2, GGA, Auxilin, Stonin 2, and Epsin²³. The IDR of proteins have classically been ignored as functionally relevant peptide sequences, but their ability to bind multiple partners illustrates just one aspect of their importance. Pan1 is known to be phosphorylated in the predicted IDR between its two EH domains²⁰. Pan1 also binds the SH3 domain of the type I myosins, Myo3 and Myo5, through its C-terminal proline-rich domain (PRD) containing IDR^{12,13}.

Circular dichroism, boiling/pelleting experiments, limited proteolysis, and quenching data validated the predicted unstructured nature of these regions. While the CD experiments confirmed the secondary structure predictions, it was surprising that the central region, containing predicted coiled-coil domains, did not pellet after boiling; however, there are several possible explanations for this result. First, the predictions of the coiled-coil domains

could be incorrect, and the region could be almost entirely unstructured but subject to self-interaction through other means. Alternatively, the structure could be extremely stable and impervious to pelleting after boiling. Finally, the boiled, unfolded peptide could re-fold into the coiled-coil structured domains and remain resistant to pelleting. Attempts to conduct circular dichroism analysis for this region were unsuccessful, and further characterization of this region will be necessary to classify it as a bona fide IDR. The C-terminus is characteristic of an IDR, being resistant to pelleting after boiling with a CD spectrum indicating mostly random coil (Figure 2A,C). This fragment, however, has a proteolytically protected fragment (Figure 2B) that may contain a structural region of interest.

The K_{SV} observed for Pan1 and the Pan1 single tryptophan mutants appears to be intermediate, indicative of each tryptophan being located in a protein region that is not fully solvent accessible. Although it may be presumed that a tryptophan in an IDR will yield a high K_{SV} , this has not been reported for the disordered region of p21Cip1, where the K_{SV} = 3.99⁴¹. Thus, the third and fourth tryptophans found in the central and C-terminus could be in an IDR of the protein and yet still be somewhat solvent inaccessible.

Through the fluorescence spectroscopic analysis of single tryptophan mutants, we now have an intriguing model for the behavior of the purified Pan1 protein. The Pan1 protein as a whole and each of the mutants have a similar K_{SV} using acrylamide as a quencher, between 4.5 and 5.4. Each of these tryptophans is accessible to acrylamide but not as accessible as a free tryptophan amino acid in solution. The emission maxima and K_{SV} values obtained for the four single-tryptophan mutants are common for tryptophans in a number of proteins, including nuclease and monellin³⁶.

Differences between the K_{SV} values for iodide and cesium quenching further illustrate that these tryptophan residues, and the protein regions in general, are fairly independent of each other. The charged residues in the vicinity of the first and fourth tryptophan are responsible for the increase in K_{SV} using iodide and cesium, respectively (see Figure 8B,C). Indeed, the decreased level of fluorescence seen for the fourth tryptophan may be due to quenching by adjacent protonated carboxyl groups. The tryptophan in the first EH domain generally shows less fluorescence intensity than the tryptophan in the second EH domain, and this could be due to quenching by proton transfer from nearby charged amino groups. Tryptophan residues are also subject to quenching through electron transfer to the peptide bonds in the protein backbone, which could also affect the emission intensity depending on the positioning of the tryptophan^{42,43}. These results emphasize the attention that should be paid when interpreting quenching data obtained using charged quenchers.

Both the first and third time-resolved anisotropy exponential terms shown in Table III provide insight into the conformational dynamics of Pan1. The presence of a 0.35 ns exponential of considerable amplitude in the time-resolved anisotropy of Trp¹²⁸⁰ reveals that the conformational dynamics in the environment of this residue is characteristic of a disordered protein region. The first and second tryptophans, as accessible residues in the binding pocket of EH domains, have time-resolved anisotropy curves that can be fitted using only two distinct exponentials. This is indicative of regions with tightly packed residues that do not allow the free spin of tryptophan, which is expected for the EH domains.

The estimates of the sizes of the Pan1 regions containing the tryptophans are also made with the assumption that the protein sizes of the tryptophan mutants are similar, as the migration by SDS-PAGE would suggest (Figure 6A), and have roughly similar levels of aggregation or self-interaction, which should be detected by velocity sedimentation (Figure 6C). If aggregation were increased in the fourth tryptophan mutant, yet somehow missed in our

sedimentation studies, this would explain the large change in predicted molecular mass for this region of the protein.

The work presented here shows that Pan1 is a protein that self-interacts through its central domain and possibly has other regions of intra-molecular self-association, as has been predicted by others²⁷. Despite numerous regions of predicted disorder, the calculated sizes of Pan1 regions containing each of the four tryptophans were fairly large. Part of this may be explained by the assumptions that are made for calculations of these molecular masses. Due to a lack of structural information of Pan1, the molecular masses were estimated from the time-resolved anisotropies under the assumptions that the domains were spherical and rotated independently; it would be impossible to estimate the molecular masses of the domains without either these assumptions or the actual structure of Pan1.

Pan1, an essential protein that acts as a hub and an endocytic scaffold, has numerous binding partners and undergoes phosphorylation-dependent regulation. For further physiologically meaningful biophysical and biochemical analyses of this protein, altering the phosphorylation state or adding Pan1 binding partners may be necessary, as well as creating mutants that disrupt Pan1-Pan1 interactions. The work presented here provides a characterization of the domain organization of Pan1 and its multitude of self-interactions that will aid in determining mechanisms that govern the spatio-temporal regulation of endocytic events. Further work will be necessary to complete the picture of the molecular mechanisms involved as Pan1 plays its role in endocytosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Pan1 contains several region of predicted disorder. A) Domain structure of Pan1, showing N-terminal region with two EH-domains (red), central region in yellow with predicted coiled coil regions (light green and green), and predicted unstructured C-terminus with a proline-rich domain (blue). Predicted coiled coils are at amino acids 687–700, 790–805, 905–918, 1020–1043 (light green), and at amino acids 1131–1190, corresponding to the extended coiled-coil domain (green). Regions of predicted random coil are shown below (white bars). Only extended regions > 40 aa without structural predictions are shown, in order to distinguish between long unstructured regions and short unstructured loops. B) The POND plot of Pan1 shows predicted disorder for the central domain and C-terminus^{24–26}.

FIGURE 2.

Pan1 is unstructured, except for the EH domains at the N-terminus. A) After boiling at 95°C for 10 minutes, the N-terminus pellets, but remains in the supernatant when a control sample is kept on ice. The central region appears to be mostly unstructured, as similar fractions of the protein pelleted both after either boiling or ice incubation, possibly due to self-interaction of this region. Pan1 C-terminus remains in the supernatant in both conditions, presumably due to the unstructured nature of this region. B) Limited proteolysis with chymotrypsin. 1) chymotrypsin alone, 2/3) His₆-Pan1¹⁵⁹⁰⁻⁷⁰⁰ (expected MW: 16 kDa, observed MW: 17 kDa), 4/5) His₆-Pan1¹²³⁰⁻⁷⁰⁰ (eMW: 50 kDa, oMW: 50 kDa), 6/7) Pan1⁶⁸⁹⁻¹¹⁹⁰-TAP (eMW: 80 kDa, oMW: 96 kDa), 8/9) His₆-Pan1¹¹⁹¹⁻¹⁴⁸⁰ (eMW: 31 kDa, oMW: 40 kDa). Differences in the expected and observed molecular weights are due to the amino acid content of the proteins, such as poly-proline region in His₆-Pan1¹¹⁹¹⁻¹⁴⁸⁰. The highest band in lane 6 (~200 kDa) is most likely high order Pan1 oligomers. Lanes 1, 3, 5, 7, and 9 contain 0.1 M chymotrypsin. C) Circular dichroism of Pan1 shows an N-terminal domain that contains some α -helical secondary structure while the C-terminus is mostly random coil.

FIGURE 3.

The Pan1 central region exists as a large molecular mass species. Through A) gel filtration and B) velocity sedimentation, Pan1⁶⁸⁹⁻¹¹⁹⁰ was determined to have a Stokes radius of 74 Å and a Svedberg coefficient of 4.5 S, giving an apparent molecular mass of 139kDa, twice the predicted molecular mass of this fragment (76.9kDa). Standards in A): thyroglobulin, ferritin, bovine serum albumin, and ribonuclease A; standards in B): thyroglobulin, catalase, lactate dehydrogenase, and bovine serum albumin.

FIGURE 4.

Amino acids 705–848 are critical for Pan1 self-interaction. A) Pan1 fragments of different lengths were GST-tagged, immobilized on glutathione-agarose beads, and probed with an anti-GST antibody. B) Pan1-TAP fragments containing various lengths of the central region bound these beads with different efficiency. Pan1-TAP fragments were incubated with glutathione-agarose beads containing bound GST-tagged constructs. These beads were then washed and probed with an antibody that recognizes the TAP-tag.

FIGURE 5.

Tryptophan fluorescence emission spectra reveal the degree of Pan1 solvent-accessibility. A) Pan1 schematic showing the location of its four endogenous tryptophans. The first two tryptophans are each in an EH domain (the second of which binds NPF motifs), the third is in the central region containing predicted coiled-coil domains, and the fourth is critical for stimulation of Arp2/3 complex activity. B) Purification of Pan1²³¹⁻¹⁴⁸⁰-TAP from yeast. Protein was purified using affinity to IgG-sepharose, TEV protease cleavage, affinity to calmodulin-sepharose beads, and eluted using 4 mM EGTA. Protein was run on an SDS-PAGE gel and stained with Gel Code Blue.

FIGURE 6.

Pan1 Tryptophan mutants behave like wild-type protein. A) Pan1 mutants containing a single tryptophan, with the other three tryptophans mutated to phenylalanine, were TAP-tagged and purified from yeast. Protein was run on an SDS-PAGE gel and stained with Gel Code Blue. B) Each of these mutants, and a tryptophan null mutant, complemented a *pan1* strain grown at 30°C (and 37°C, not shown). C) Velocity sedimentation of the mutants and wild-type protein is similar, indicating that these proteins are similar in solubility and aggregation.

FIGURE 7.

Pan1 Tryptophan mutants allow for a more refined analysis of Pan1 structure. A) Emission spectra of the four Pan1 single-tryptophan mutants when excited at 295nm. The majority of the tryptophan emission intensity for the wild-type protein is contributed by the second tryptophan. B) When normalized and compared with the wild-type protein, the peak of the emission spectra of the first and third tryptophan are shifted to shorter wavelengths and the peak of the fourth tryptophan spectrum is shifted to longer wavelengths.

FIGURE 8.

Quenching of the tryptophan mutant proteins reveals electrostatic effects. Stern-Volmer plots of quenching using A) acrylamide ($n = 2$), B) iodide ($n = 3$), and C) cesium ($n = 2$) are shown, with histograms of the Stern-Volmer constants (K_{SV}) to the right of each plot (error bars represent $\pm$ SD). The initial slope of the line (----) was used when curvature in the plots was observed. Acrylamide quenching experiments reveal moderate solvent accessibility of all four tryptophans and the linear nature of each Stern-Volmer plot is indicative of collisional quenching playing the dominant role. Iodide and cesium quenching shows differential K_{SV} for tryptophans in environments surrounded by charged amino acids.

FIGURE 9.

Time-resolved polarized fluorescence of Pan1 tryptophan mutants W312 and W642. Polarized fluorescence of Pan1 variants W312 and W642, is shown in panels A) and B), respectively. In these panels the left and the right halves depict the time-correlated single photon counting (TCSPC) data collected through a vertical and horizontal polarizer, respectively, upon excitation by vertically polarized light. In the main part of each half-panel the dots represent TCSPC data, while the solid line represents the best fit by the model function. The strip under the main part depicts the weighted residuals, *i.e.*, the channel-by-channel differences between the data and the fit divided by the standard deviation for the given channel. The inset in the top right corner of each half-panel depicts the autocorrelation of unweighted residuals, peak-normalized to 1.

FIGURE 10. Time-resolved polarized fluorescence of Pan1 tryptophan mutants W957 and W1280. Polarized fluorescence of Pan1 variants W957 and W1280, is shown in panels A) and B), respectively. The data are represented as in Figure 9.

FIGURE 11.

Compilation of time-resolved polarized anisotropy for Pan1 tryptophan mutants. Data collected in Figures 9 and 10 were reconstructed as sums of exponentials using the best-fit model parameters. This Figure reveals differences in the amplitudes of the initial drops ($W1280 > W957 > W312 > W642$) and in the final slopes ($W312 > W642 > W957 > W1280$), indicative of the conformational dynamics in the local environment of the tryptophan residue and the overall size of the domain containing that tryptophan, respectively.

TABLE I

Bimolecular rate constants of Pan1 single tryptophan mutants.

Pan1 mutant	K_{SV} (M^{-1})	τ_0 (ns)	k_q ($M^{-1} \times s^{-1}$)
W312	$K_{SV(acryl)}$: 4.9 ± 0.1 $K_{SV(KI)}$: 6.7 ± 0.3 $K_{SV(CsCl)}$: 0.5 ± 0.1	1.58	$k_q(acryl)$: 3.1×10^9 $k_q(KI)$: 4.3×10^9 $k_q(CsCl)$: 3.2×10^8
W642	$K_{SV(acryl)}$: 4.5 ± 0.2 $K_{SV(KI)}$: 2.0 ± 0.5 $K_{SV(CsCl)}$: 0.3 ± 0.1	2.94	$k_q(acryl)$: 1.5×10^9 $k_q(KI)$: 6.8×10^8 $k_q(CsCl)$: 1.0×10^8
W957	$K_{SV(acryl)}$: 5.1 ± 0.1 $K_{SV(KI)}$: 2.6 ± 0.2 $K_{SV(CsCl)}$: 1.0 ± 0.2	1.59	$k_q(acryl)$: 3.2×10^9 $k_q(KI)$: 1.6×10^9 $k_q(CsCl)$: 6.3×10^8
W1280	$K_{SV(acryl)}$: 5.4 ± 0.1 $K_{SV(KI)}$: 2.3 ± 0.2 $K_{SV(CsCl)}$: 2.5 ± 0.2	1.65	$k_q(acryl)$: 3.3×10^9 $k_q(KI)$: 1.4×10^9 $k_q(CsCl)$: 1.5×10^9

TABLE II

Parameters describing total fluorescence emission decay of Pan1 single tryptophan mutants.

Pan1 mutant	(ns)	% Intensity	%	-weighted mean
W312	0.02	0.008	0.67	1.58
	0.25	0.006	0.03	
	1.15	0.035	0.05	
	3.88	0.249	0.10	
	7.70	0.703	0.14	
W642	0.04	0.003	0.22	2.94
	0.34	0.012	0.10	
	1.20	0.048	0.12	
	3.44	0.348	0.30	
	6.53	0.589	0.27	
W957	0.03	0.008	0.44	1.59
	0.30	0.020	0.11	
	1.19	0.107	0.14	
	3.39	0.451	0.21	
	6.76	0.413	0.10	
W1280	0.03	0.008	0.39	1.65
	0.32	0.023	0.12	
	1.21	0.118	0.16	
	3.35	0.502	0.25	
	6.70	0.349	0.09	

TABLE III

Parameters describing time-resolved anisotropy of Pan1 tryptophan mutants

Pan1 mutant	(ns)	Absolute	%
W312	0.03	0.20	0.45
	3.45	0.03	0.06
	22.00	0.22	0.49
W642	0.11	0.03	0.10
	4.07	0.05	0.16
	41.48	0.21	0.73
W957	0.53	0.03	0.12
	3.09	0.05	0.20
	69.83	0.17	0.68
W1280	0.35	0.05	0.19
	2.05	0.07	0.28
	119.57	0.14	0.54