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Disordered TPPP/p25 binds GTP and displays Mg^{2+}-dependent GTPase activity

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ABSTRACT

The disordered Tubulin Polymerization Promoting Protein/p25 (TPPP/p25) modulates the dynamics and stability of the microtubule system and plays a crucial role in differentiation of oligodendrocytes. Here we first demonstrated by multinuclear NMR that the extended disordered segments are localized at the N- and C-terminals straddling a flexible region. We showed by affinity chromatography, fluorescence spectroscopy and circular dichroism that GTP binds to TPPP/p25 likely within the flexible region; neither positions nor intensities of the peaks in the assigned terminals were affected by GTP. In addition, we demonstrated that TPPP/p25 specifically hydrolyses GTP in an Mg^{2+}-dependent manner. The GTPase activity is comparable with the intrinsic activities of small G proteins suggesting its potential role in multiple physiological processes.

1. Introduction

The majority of intracellular proteins have well-defined secondary and tertiary structures; however, predictions based on the in silico analysis of the human genome, some of them proved experimentally, have suggested that a significant portion of the proteins does not feature predictable 3D structure [1]. These proteins denoted as *intrinsically disordered* or *unstructured* proteins contain at least one experimentally determined unstructured region [2]. Despite the lack of a well-defined structure, *intrinsically disordered* proteins and disordered regions carry out important biological functions, being typically involved in regulation, signaling and control; they seldom display intrinsic catalytic activity [1,3,4]. Their functions are linked mostly to their macromolecular assemblies, which frequently cause formation of stable aggregates. Many of them are tightly linked to the development of different neurodegenerative diseases and function as hallmark proteins [4].

Tubulin Polymerization Promoting Protein/p25 (TPPP/p25) was isolated and identified as a brain-specific protein, the primary target of which is the microtubule system [5] and it was designated as Tubulin Polymerization Promoting Protein on the basis of its in vitro and in vivo functions [6]. It is expressed endogenously mainly in oligodendrocytes; it seems to control the dynamics and stability of the microtubule system as a microtubule-associated protein [7]. TPPP/p25 expression is crucial for the differentiation of oligodendrocytes in rearrangement of the microtubule network during the projection elongation prior to the onset of myelination. In addition, we have also postulated that TPPP/p25 promotes the specific acetylation of α-tubulin affecting the microtubule-derived cell motility [8].

We have reported based on circular dichroism (CD), fluorescence spectroscopy, limited proteolysis and ^{1}H NMR studies that TPPP/p25 belongs to the family of the intrinsically disordered proteins; predictions established that the N-terminal segment of TPPP/p25 is unfolded [9,10]. More recently, multinuclear NMR studies have showed that the C-terminal part of the human paralogue TPPP3/p20, which is a truncated, N-terminal-free form of TPPP/p25, is "conformationally disordered" [11]. Previously, we showed that GTP modulates the binding of TPPP/p25 to tubulin resulting in the inhibition of TPPP/p25-induced tubulin polymerization [5]. In addition, we found that GTP suspended the influence of TPPP/p25 on microtubule-derived mitosis in *Drosophila* embryos expressing tubulin-GFP fusion protein [6].

Abbreviations: CD, circular dichroism; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; TPPP/p25, Tubulin Polymerization Promoting Protein/p25

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In this paper we show by multinuclear NMR that the unstructured segments of TPPP/p25 are localized at the N- and C-terminal tails and straddle a flexible segment which contains potential GTP binding motifs, and that GTP, indeed, binds to TPPP/p25, which displays Mg^{2+}-dependent GTPase activity.

2. Materials and methods

2.1. TPPP/p25 purification

Human recombinant TPPP/p25 possessing a His-tag tail at N- or C-terminus was expressed in *Escherichia coli* BL21 (DE3) cells and isolated as described previously [9]. Comparative studies performed with the preparations showed virtually no difference either in the structural or in the interacting features of TPPP/p25 depending on the presence or the position of the His tag on the human recombinant protein.

2.2. Protein determination

The protein concentration was measured by the Bradford method [12] using the Bio-Rad protein assay kit.

2.3. Nucleotides

All nucleotides were purchased from Sigma–Aldrich (St. Louis, MO, USA). Nucleotide concentration was determined by UV–visible absorption spectroscopy by using the following molar extinction coefficients: for GTP and GDP $\varepsilon_{253} = 13\,700\ M^{-1}\ cm^{-1}$, and for ATP $\varepsilon_{259} = 15\,400\ M^{-1}\ cm^{-1}$.

2.4. Assignment by NMR spectroscopy

Uniformly ^{15}N-labeled TPPP/p25 was produced with the same purification procedure used for the unlabeled proteins, using M9 minimal medium containing $^{15}NH_4Cl$ as sole nitrogen source or $^{15}NH_4Cl$ and ^{13}C-glucose. Solutions containing single or double labeled TPPP/p25 (400 μM) in 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 6.8 were used for backbone assignment by NMR. Spectra were acquired at 300 K using home-built 750 and 950 MHz NMR spectrometers, controlled with GE/Omega software and equipped with an Oxford Instruments Company magnet and a home-built triple-resonance pulsed-field-gradient probehead. Resonance assignments have been obtained from 3D triple-resonance HNCA, (H)CC(CO)NH NMR experiments performed on a ^{13}C, ^{15}N uniformly labeled sample. Sequential assignments were confirmed – where possible – with 3D ^{15}N-edited TOCSY-HSQC and NOESY-HSQC measurements using a ^{15}N uniformly labeled sample. GTP binding studies were performed on a Bruker DRX500 instrument equipped with a 5 mm inverse probe-head using a ^{15}N labeled sample. 2D HSQC measurements were carried out by 8 scans. All spectra were processed using the NMRPipe/nmrDraw software [13] and analyzed by CARA program, while 1D and 2D spectra visualization was achieved with TOPSPIN and with the public domain graphics program Sparky.

2.5. Affinity chromatography of TPPP/p25 on γ-amino-hexyl-GTP-Sepharose® screening column

Hundred micrograms of recombinant TPPP/p25 was applied to a 0.2 ml column of GTP-Sepharose (degree of substitution: 5 μmol GTP/ml gel) (Jena Bioscience) equilibrated with 25 mM NaCl, 20 mM Tris, pH 7.0, and the flow-through fractions (200 μl) were collected at 4 °C. The matrix was washed with an additional 0.6 ml of buffer. The retained protein was eluted with 17.4 mM, 0.4 ml of GTP solution, and fractions (200 μl) were collected. The flow-through and eluate fractions were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), separated on a 13.5% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 containing beta-mercaptoethanol and dithioerythritol.

2.6. Fluorescence spectroscopy

Fluorescence spectra were measured on a FluoroMax-3 spectrofluorometer (Jobin Yvon Inc., Longiumeau, France), using 1 cm thermostated cuvettes at 25 °C. Scanning was repeated three times, and the spectra were averaged, the buffer spectrum was subtracted in each case. Intrinsic tryptophan fluorescence spectra of TPPP/p25 (2.4 or 8 μM) were recorded at an excitation wavelength of 290 nm (slit width 10 nm) in 10 mM Tris buffer pH 7.2. Emission spectra were collected from 300 to 400 nm (slit width 2 nm). Quenching experiments with nucleotides (MgGTP and MgATP) were performed by addition of small aliquots of concentrated stock solutions to the cuvette.

2.7. CD spectroscopy

CD spectra were acquired with a Jasco J-720 spectropolarimeter (Tokyo, Japan) in the 190–260 nm wavelength range employing 0.1 cm thermostated cuvettes at 25 °C using 20 mM Tris buffer, pH 7.2. Scanning was repeated three times at scan speed 20 nm/min with step size of 0.2 nm, and the spectra were averaged. 7.5 μM TPPP/p25 were titrated with (Mg)GTP or (Mg)ATP in the concentration range of 50–1000 μM. The reaction mixtures (200 μl) were incubated for 5 min at 25 °C before recording the spectra.

2.8. Malachite green phosphate release assay

The inorganic phosphate produced by TPPP/p25-catalyzed GTP hydrolysis was detected as described in [14]. This assay is based on quantification of the highly colored complex of phosphomolybdate and malachite green. The absorbance was measured at 660 nm by using a precision microplate reader (Wallac 1420, Perkin–Elmer). The reaction mixture containing 80 μM freshly prepared TPPP/p25 was incubated with 1500 μM nucleotide (GTP, GDP or ATP) in 20 mM Tris buffer, pH 7.4, containing 5 mM $MgCl_2$ or 5 mM Mn^{2+} or 100 mM NaCl as indicated, at room temperature. Aliquots (25 μl) were withdrawn at different incubation times and added to 100 μl of malachite green reagent to terminate the enzyme reaction. The malachite green reagent was prepared from stock solutions of ammonium molybdate (5.72%, w/v in 6 N HCl), polyvinyl alcohol (2.32%, w/v), malachite green (0.0812%, w/v), and distilled water mixed at a ratio of 1:1:2:2, respectively. Control measurements were carried out with TPPP/p25 or nucleotide solutions alone. The control MgGTP hydrolysis was subtracted from that measured in the mixture of TPPP/p25 and MgGTP. P_i calibration curve was determined with KH_2PO_4 standard.

2.9. GTP hydrolysis followed by NMR spectroscopy

Two-hundred micrograms of freshly prepared TPPP/p25 solution was typically used in 50 mM Tris buffer, pH 7.0, containing 4 mM $MgCl_2$ and 1360 μM GTP. All NMR measurements were performed at 300 K. GTPase activity was followed by ^{31}P NMR measurements at 101.25 MHz, on a Bruker Avance 250 MHz instrument, equipped with a 5 mm $^{13}C/^{19}F/^{31}P$ probe-head. 1D inverse gated ^{1}H decoupled spectra were collected using a 50 ppm spectral window, the number of scans varied between 3000–6000, a 30° pulse and a relaxation delay of 2 s was applied. The