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Microtubule assembly-derived by dimerization of TPPP/p25. Evaluation of thermodynamic parameters for multiple equilibrium system from ITC data

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ABSTRACT

Background: The disordered Tubulin Polymerization Promoting Protein/p25 (TPPP/p25) modulates the dynamics and stability of the microtubule system. In this paper the role of dimerization in its microtubule-related functions is established, and an approach is proposed to evaluate thermodynamic constants for multiple equilibrium systems from ITC measurements.
Methods: For structural studies size exclusion chromatography, SDS-PAGE, chemical cross-linking, circular dichroism, fluorescence spectroscopy and isothermal titration calorimetry were used; the functional effect was analyzed by tubulin polymerization assay. Numerical simulation of the multiple equilibrium was performed with Mathematica software.
Results: The dimerization of TPPP/p25 is promoted by elevation of the protein concentration and by GTP addition. The dimeric form displaying enhanced tubulin polymerization promoting activity is stabilized by disulfide bond or chemical cross-linking. The GTP binding to the dimeric form ($K_{d\text{-}GTP} = 200\ \mu M$) is tighter with one order of magnitude than to the monomeric one leading to the enrichment of the dimers. A mathematical model elaborated for the multiple equilibrium of the TPPP/p25-GTP system was validated by fitting the GTP-dependent changes of ellipticity and fluorescence signal in the course of TPPP/p25 titrations. The evaluation of the equilibrium constants rendered it possible to determine the thermodynamic parameters of the association of different TPPP/p25 forms with GTP from ITC measurements.
Conclusions/General Significance: The dimerization of TPPP/p25 with favorable physiological functional potency is proposed to play significant role in the fine tuning of TPPP/p25-mediated microtubule assembly; the unfolded monomers might be involved in the formation of pathological inclusions characteristic for Parkinson's disease and other synucleinopathies.

1. Introduction

Intrinsically disordered or natively unfolded proteins, which do not have well-defined 3D structures or contain at least 30–40 amino acid long disordered segment(s), are rather common in living cells and fulfill essential functions such as regulation of transcription and translation or cellular signal transduction [1,2]. These proteins play crucial role in the etiology of conformational diseases such as Parkinson's and Alzheimer's diseases [3,4]. Tubulin Polymerization Promoting Protein/p25 (TPPP/p25) was identified as a disordered protein, its primary target is the microtubule system, and the protein was designated on the basis of its in vitro [5] and in vivo functions [6]. In normal human brain TPPP/p25 is expressed predominantly in oligodendrocytes where it plays indispensable role in their differentiation and the ensheathment of axons, likely via its role in the rearrangement of the microtubule network during the projection elongation prior to the onset of myelination [7,8]. TPPP/p25 regulates the dynamics and stability of the microtubule system as a microtubule-associated protein [9], and promotes the specific acetylation of α-tubulin on residue Lys-40 affecting the dynamics of the microtubule system as well as the microtubule-derived cell motility [10]. The intracellular concentration of the protein can be controlled at protein level by the proteasome machinery as well as at posttranscriptional level by specific microRNA [8,11]. In pathological conditions, TPPP/p25 was found to be enriched and co-localized with alpha-synuclein in neuronal and glial inclusions in the case of Parkinson's disease and multiple system atrophy,

Abbreviations: CD, Circular dichroism; DTE, Dithioerythritol; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); ITC, Isothermal titration calorimetry; ME, 2-mercaptoethanol; PAGE, Polyacrylamide gel electrophoresis; PDM, N,N′-1,3-phenylenedimaleimide; SEC, Size exclusion gel chromatography; SDS, Sodium dodecyl sulfate; TPPP/p25, Tubulin Polymerization Promoting Protein/p25; Tris, Tris(hydroxymethyl)aminomethane

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respectively [12]; therefore it was proposed to be a marker of synucleinopathies [12,13].

Recently we have shown that the extended disordered segments of TPPP/p25 are localized at the N- and C-terminals, straddling a flexible region [14]. The protein contains four out of the five representative GTP binding motifs characteristic for small G proteins. Indeed, we have shown by affinity chromatography, fluorescence and circular dichroism (CD) spectroscopies that TPPP/p25 binds GTP likely within the middle, flexible region and the EC_{50} value of the GTP binding to TPPP/p25 was estimated to be around 500 μM [14]. We also provided evidence that TPPP/p25 specifically hydrolyses GTP in a Mg^{2+}-dependent manner, its activity is comparable with the intrinsic activities of other small G proteins [14].

In this paper we demonstrate that isolated human recombinant TPPP/p25 occurs in monomeric and homodimeric forms and the binding of GTP to these two species is distinct, shifting the equilibrium towards the dimer formation with functional consequences. A model has been elaborated for the characterization of the multiple equilibrium in the complex TPPP/p25-GTP system.

2. Materials and methods

2.1. TPPP/p25 purification

Human recombinant TPPP/p25 possessing His-tag tail at N- or C-terminus was expressed in *E. coli* BL21 (DE3) cells and isolated as described previously [12].

2.2. Determination of protein and nucleotide concentration

The protein concentration was measured by the Bradford method [15] using the Bio-Rad protein assay kit. Nucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nucleotide concentration was determined by UV/visible absorption spectroscopy by using the molar extinction coefficient for GTP and GDP $\varepsilon_{253} = 13700\ M^{-1}\cdot cm^{-1}$. The molar extinction coefficient for TPPP/p25 is $\varepsilon_{280} = 9970\ M^{-1}\cdot cm^{-1}$.

2.3. Cross-linking

Cross-linking was carried out at 30 °C for 60 min with N,N′-1,3-phenylenedimaleimide (PDM) in phosphate buffered saline (10 mM phosphate buffer pH 7.4 containing 120 mM NaCl). Final concentration of the protein was 200–400 μM, while the ratio of the TPPP/p25 and PDM was always kept at 1:2. The cross-linking was initiated by the addition of PDM and terminated by 50 mM dithioerythritol (DTE). Control and cross-linked TPPP/p25 samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions (in the presence of 2-mercaptoethanol (ME) and DTE), and stained with Coomassie Brilliant Blue R-250 according to Laemmli [16].

2.4. Size exclusion gel chromatography (SEC)

ÄKTA purifier FPLC system (GE Healthcare, formerly Amersham Biosciences) was used with a 24 ml Superose 12 10/300 GL column (GE Healthcare) at a flow-rate of 0.5 ml/min for chromatography. The column was equilibrated at room temperature with 20 mM Tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.2, containing 150 mM NaCl, and 500 μM GTP plus 3 mM $MgCl_2$ where indicated. The buffer solutions were filtered through a 0.45 μm membrane filter (Millipore). 200 μl TPPP/p25 solutions were loaded to the column at different concentrations. TPPP/p25 treated with the cross-linker PDM or incubated with MgGTP for 60 min at room temperature was also used for SEC experiments. Flow-through fractions were recorded at 280 and 254 nm. The Microcal Origin 7.0 software was used to calculate the integrated areas of the peaks. The partition coefficient (K_{av}) was determined for the observed peaks according to the following equation:

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (1)$$

where V_e represents the elution volume, V_0 is the void volume, and V_t is the total column volume. For the calibration of the column the following globular proteins were used (Stokes' radius, R_S/molecular weight, Mw): Cytochrome C, Horse Heart (17.4 Å/12.5 kDa), Chymotrypsinogen A, Bovine Pancreas (20.9 Å/25.0 kDa), Carbonic Anhydrase, Bovine Erythrocyte (24.4 Å/29 kDa), Ovalbumin, Chicken Egg (30.5 Å/43 kDa), Albumin, Bovine Serum (35.5 Å/66.0 kDa), and Aldolase, Rabbit Muscle (48.1 Å/158 kDa). The apparent molecular mass of the observed protein peaks was determined from a calibration curve constructed by plotting K_{av} values versus log(Mw). The Stokes' radius of the observed protein peaks was evaluated from a calibration curve constructed by plotting $(-\log(K_{av}))^{1/2}$ values versus R_S. The theoretical Stokes' radii for the different conformations of proteins with molecular mass of 24.8 kDa (monomer) and 49.6 kDa (dimer) were calculated according to the following empirical equations [3]:

$$\log\left(R_s^{N}\right) = -(0.204 \pm 0.023) + (0.357 \pm 0.005) * \log(Mw) \quad (2)$$

$$\log\left(R_s^{MG}\right) = -(0.053 \pm 0.094) + (0.334 \pm 0.021) * \log(Mw) \quad (3)$$

$$\log\left(R_s^{U}\right) = -(0.723 \pm 0.033) + (0.543 \pm 0.007) * \log(Mw) \quad (4)$$

$$\log\left(R_s^{NU-coil}\right) = -(0.551 \pm 0.032) + (0.493 \pm 0.008) * \log(Mw) \quad (5)$$

$$\log\left(R_s^{NU-PMG}\right) = -(0.239 \pm 0.055) + (0.403 \pm 0.012) * \log(Mw) \quad (6)$$

Here R_s^N, R_s^{MG} and R_s^U are the Stokes' radii of a globular protein with the given molecular mass in its native (N), molten globule (MG) and unfolded (U) conformation, respectively. $R_s^{NU\text{-}coil}$ and $R_s^{NU\text{-}PMG}$ correspond to the Stokes' radii of natively unfolded coils and natively unfolded premolten globules.

2.5. Determination the number of SH groups

The number of SH groups in TPPP/p25 was determined by using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma D8130) reagent. The assay was carried out at room temperature, in 100 mM Tris buffer pH 8.0 containing 10 mM ethylenediaminetetraacetic acid; the final concentrations of the protein and the DTNB were 40 μM and 250 μM, respectively. Known amount of DTE was used for calibration, and absorbance was read at 412 nm with a Wallac Victor2 multiplate reader (PerkinElmer, LifeSciences).

2.6. 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 3 times at sensitivity set to 100 mdeg and scan speed 20 nm/min with step size of 0.2 nm, and the spectra were averaged. 7.5 μM TPPP/p25 was titrated with MgGTP in the concentration range of 50–1000 μM. The reaction mixtures were incubated for 5 min at 25 °C before recording the spectra.

2.7. Fluorescence spectroscopy

Fluorescence spectra were measured on a FluoroMax-3 spectrofluorometer (Jobin Yvon Inc., Longiumeau, France), using 1 cm thermostated