REPORT

The polypyrimidine tract binding protein is a monomer

TOM P. MONIE,¹ HELENA HERNANDEZ,³ CAROL V. ROBINSON,³ PETER SIMPSON,² STEPHEN MATTHEWS,² and STEPHEN CURRY¹

¹Division of Cell and Molecular Biology and ²Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, United Kingdom

³Department of Chemistry, University of Cambridge, Cambridge CB2 1EW, United Kingdom

ABSTRACT

The polypyrimidine tract binding (PTB) protein is a potent regulator of alternative mRNA splicing. It also participates in other essential cellular functions, including translation initiation and polyadenylation. Several published reports have suggested that the protein forms a dimer in solution, a feature that has been widely incorporated into mechanistic models of protein function. However, recent studies have provided indications that full-length PTB is a monomer. Here we present new biophysical and biochemical evidence supporting the monomeric status of the protein. By use of blue-native polyacrylamide gel electrophoresis and size-exclusion chromatography, PTB was observed as a single molecular species under native reducing environments, though in oxidizing conditions, a larger protein species was also detected. Further analyses of wild-type and mutant PTB molecules with SDS-PAGE and time-of-flight electrospray ionization mass spectroscopy confirmed these observations. They also identified the single reduced species as monomeric PTB and the higher-molecular-weight nonreduced species as disulphide-linked PTB dimer mediated by Cys23. Our results indicate that the use of oxidizing environments in previous studies is likely to have contributed to the misassignment of PTB as a dimer. Although purified PTB may form disulphide-linked dimers under these conditions, in the reducing intracellular environment the protein will be monomeric. These findings have implications for the construction of models of PTB function in regulating mRNA metabolism.

Keywords: polypyrimidine tract binding protein; PTB; monomer; disulphide; dimer

INTRODUCTION

The polypyrimidine tract binding protein (PTB) is an RNA-binding protein with an extensive repertoire of cellular roles. It has been demonstrated to play a crucial role in the negative regulation of alternative splicing, via the repression of exon inclusion, for a growing number of genes (see Izquierdo et al. 2005; Sharma et al. 2005; and references therein). Indeed, PTB has been shown to regulate its own alternative splicing (Wollerton et al. 2004). PTB also promotes translation initiation from a variety of viral (Hellen et al. 1993; Kaminski et al. 1995; Pilipenko et al. 2001) and cellular (Mitchell et al. 2001, 2005; Pickering et al. 2003) internal ribosome entry sites and is reported to regulate the polyadenylation (Luo 1999; Castelo-Branco et

al. 2004), localization (Cote et al. 1999), and stabilization (Knoch et al. 2004) of mRNA.

PTB contains four RNA recognition motifs (RRMs), the most common motif found in RNA-binding proteins (Maris et al. 2005). In PTB, both RRM1 and 4 display a classic $\beta\alpha\beta\beta\alpha\beta$ topology, while RRM2 and 3 possess a C-terminal extension incorporating a fifth β -strand, which enlarges the RNA-binding β -sheet surface (Conte et al. 2000; Simpson et al. 2004). Each RRM in PTB contributes to RNA binding, with specificity for short polypyrimidine sequences, although the protein has also recently been reported to associate with double-stranded RNA (Mitchell et al. 2005).

Initial investigations of the oligomerization state of PTB were interpreted as indicating that PTB is a homodimer in solution, with dimerization being postulated to result from interactions predominantly involving RRM2 (Perez et al. 1997; Oh et al. 1998). The assumption of homodimer formation was widely incorporated, both explicitly and implicitly, into models of PTB function (Wagner and Garcia-Blanco 2001; Mitchell et al. 2003; Pickering et al. 2004). However, more recent analytical ultracentrifugation analy-

Reprint requests to: Stephen Curry, Biophysics Section, Division of Cell and Molecular Biology, Imperial College London, South Kensington Campus, Room 746 Huxley Building, London SW7 2AZ, United Kingdom; e-mail: s.curry@imperial.ac.uk; fax: +44-20-7589-0191.

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sis of PTB and a range of recombinant subfragments suggest that the protein may in fact be monomeric (Simpson et al. 2004; Amir-Ahmady et al. 2005) and have therefore challenged the existing consensus on PTB dimerization. The methods used in these recent studies rely on an accurate determination of the PTB concentration, a nontrivial measurement given the low extinction coefficients of the constructs used (Simpson et al. 2004; Amir-Ahmady et al. 2005).

We report here new concentration-independent biochemical and biophysical analyses of wild-type and mutant PTB. Our results show that PTB dimerization only occurs in oxidizing conditions and is mediated by a single intermolecular disulphide bridge involving Cys23. This finding indicates that some of the previous reports of PTB dimerization are due to experiments being performed under oxidizing conditions. Under reducing conditions, which is the redox state relevant to the intracellular milieu, PTB is found to be monomeric.

RESULTS

PTB dimerization requires an oxidizing environment

Comparison of the range of published results on PTB oligomerization suggested that the assignment of PTB as a monomer or dimer might correlate with the use of reducing or oxidizing conditions in the experiment (Perez et al. 1997; Oh et al. 1998; Simpson et al. 2004). We therefore decided to investigate this possibility explicitly.

Blue native polyacrylamide gel electrophoresis (BN-PAGE), a “charge shift” method of electrophoresis (Schagger et al. 1994), was used to determine the molecular mass and native oligomeric status of purified recombinant PTB1. In oxidizing conditions, both monomeric and dimeric forms were observed (Fig. 1, lane 1); no species larger than a dimer was identified. However, in a reducing environment, PTB1 dimerization was completely abrogated (Fig. 1, lane 2). Interestingly, the PTB1 monomer band appeared as a doublet in the absence of DTT but as a single band following reduction (Fig. 1, cf. lanes 1 and 2).

FIGURE 1. Blue-native polyacrylamide gel electrophoresis (BN-PAGE). Ten micrograms of PTB1 was separated under native conditions using BN-PAGE. (Lane 1) Oxidizing environment, (lane 2) reducing environment (+10 mM DTT). Distinct protein bands are marked by arrows and their calculated molecular weight listed.

FIGURE 2. Size-exclusion chromatography of PTB1. Size-exclusion chromatography was performed on PTB1 under both oxidizing (solid line) and reducing conditions (dashed line). The estimated molecular weights of the elution peaks are labeled.

The apparent molecular weight of PTB1 was calculated from triplicate experiments using a linear plot of M_r against electrophoretic migration of known protein standards (see Materials and Methods). The calculated molecular weights were as follows: PTB1 dimer, 159 kDa; nonreduced PTB1 monomer, 101 and 90 kDa; and reduced PTB1 monomer, 90 kDa. While these values are larger than are the theoretical masses (monomer, 58,316 Da; dimer, 116,633 Da), the monomeric values are broadly consistent with those obtained previously by using size-exclusion chromatography (Simpson et al. 2004). It is likely that the molecular mass is overestimated by this technique due to the extended rod-shaped structure of PTB1, which was revealed by small-angle X-ray scattering (Simpson et al. 2004).

We next used size-exclusion chromatography to examine PTB oligomerization. Under reducing conditions (2 mM DTT), PTB1 eluted with an apparent molecular weight in the region of 100 kDa, with minimal additional protein signal outside this major peak (Fig. 2, dotted line; Simpson et al. 2004). This is likely to correspond to monomeric PTB given the asymmetric shape of the protein. In the absence of any reducing agent, although most of the protein eluted at ~100 kDa, the peak was significantly broader and a second elution peak was observed at a predicted molecular mass of 239 kDa (Fig. 2, solid line). This elution profile is broadly consistent with that obtained under oxidizing conditions by Perez et al. (1997). The 239-kDa peak observed in our elution under oxidizing conditions is presumably due to disulphide-linked dimers and probably corresponds to the protein shoulder previously observed at ~200 kDa (Perez et al. 1997). The predicted mass of this dimer peak is over twice that of the eluted monomeric protein and suggests that formation of an intermolecular disulphide bridge does not lead to a more compact, globular protein conformation.

Cys23 mediates intermolecular disulphide bridge formation

The change in oligomeric profile for PTB1 upon variation in reducing environment provided strong evidence that the observed dimers resulted from intermolecular disulphide bridge formation. PTB contains three cysteines: at residue 23, in the N-terminal sequence preceding RRM1, and at residues 250 and 251 within RRM2. The N-terminal sequence of PTB is likely to be flexible since it is proteolytically sensitive (Simpson et al. 2004), and Cys23 is therefore potentially accessible for participation in disulphide bridges. The three-dimensional structure of RRM2 (PDB ID 1sjr) reveals that Cys250 and Cys251 are found close to the protein surface at the C-terminal end of the loop between helix-2 and strand β 4 (Simpson et al. 2004). Although Cys251 appears inaccessible to solvent, Cys250 is partially surface-exposed and may also be able to participate in disulphide bridge formation.

To clarify whether PTB could form disulphide bridge-mediated dimers, site-directed mutagenesis was used to generate the mutant constructs PTB1-C23S and PTB1-C250S/C251S. The formation of disulphide-mediated dimers was assayed by SDS-PAGE using either an oxidizing or a reducing sample loading buffer. Under reducing conditions, wild-type PTB1 and both mutants (PTB1-C23S, PTB1-C250S/C251S) migrated solely as monomers (Fig. 3, lanes 1–3). In contrast, in an oxidizing environment, both PTB1 and PTB1-C250S/C251S migrated at sizes consistent with the presence of both monomer and dimer (Fig. 3, lanes 4,6). However, PTB1-C23S remained entirely monomeric (Fig. 3, lane 5). These results clearly demonstrated not only that PTB1 can form disulphide-mediated dimers but that these are mediated solely through intermolecular interactions involving Cys23.

The lack of formation of intermolecular disulphide bonds by cysteines C250 and C251 within RRM2 is consistent with the observation by NMR that they readily form an intramolecular connection. When exposed to oxidizing conditions (either the absence or the depletion of 10 mM

FIGURE 3. SDS-PAGE analysis of PTB1 and cysteine mutant derivatives. One microliter of 10 μ M protein samples was analyzed in either reducing (lanes 1–3) or oxidizing (lanes 4–6) environments. (Lanes 1,4) PTB1, (lanes 2,5) PTB1-C23S, (lanes 3,6) PTB1-C250S/C251S, (lane 7) Benchmark protein ladder (Invitrogen). The arrow indicates the presence of disulphide-mediated dimers.

FIGURE 4. NMR analysis of intramolecular disulphide formation by C250 and C251. (A) Expanded region from the two-dimensional ^1H - ^{15}N HSQC spectrum of PTB RRM2 domain in the presence (black) and the absence (red) of 10 mM reduced DTT. Some residues exhibiting a change in chemical shift upon oxidation are labeled. A question mark indicates the presumed shift of C251, which was not determined unambiguously. (B) Cartoon representation of PTB RRM2. Residues associated with significant shifts in A are shown in a stick representation (side chains only). The structure is colored according to the change in chemical shift upon oxidation (gray, no shift; pink, small shift; red, large shift). The figure was generated using Pymol (DeLano Scientific).

DTT), chemical shift changes are observed in both cysteines and also in residues immediately adjacent in the structure, which are entirely consistent with disulphide bond formation (Fig. 4A,B). The chemical shift changes are reversible by the addition of fresh reducing agent and are not accompanied by any change in NMR line width, indicating that the domain remains monomeric. Together these data indicate that the cysteine residues in RRM2 readily form an intramolecular disulphide bond.

To probe the oligomerization state of PTB and the impact of the Cys residues under more native conditions, we used nanospray ElectroSpray Ionisation Mass Spectrometry (ESI-MS), a technique that is well adapted to the analysis of both covalent and noncovalent protein–protein interactions (Hernandez and Robinson 2001; Hanson and Robinson 2004). In particular, we compared the oligomerization states of wild-type PTB1, the PTB1-C23S mutant, and PTB1-1234, a truncated construct that lacks the first 54 amino acids (and therefore lacks Cys23). Proteins were analyzed at a concentration of 10 μ M in 50 mM ammonium acetate (pH 6.5).

Under oxidizing conditions, analysis of PTB1 identified mass/charge peaks consistent with the presence of both monomeric ($58,328 \pm 7$ Da) and dimeric species ($116,661 \pm 15$ Da) (Fig. 5A). For the monomer, two conformations were indicated by the presence of two charge state distributions in the spectra. Increasing instrument pressure in the source/transfer region, thereby favoring noncovalent dimer formation, produced minimal detectable changes in the observed spectra. In addition, MS/MS analysis failed to dissociate the PTB1 dimer peaks into monomers, even with increasing collision energy (data not shown). However, the presence of 5 mM DTT eliminated

FIGURE 5. Electrospray ionization mass spectrometry analysis of PTB proteins. Samples containing 2 μ L of 10 μ M protein in 50 mM ammonium acetate (pH 6.5) were analyzed by nanospray electrospray ionization mass spectrometry under native conditions using a modified QToF2 instrument fitted with a standard Z-spray nanoflow electrospray source. The mass/charge peaks were identified using MassLynx, and the corresponding masses are listed to the right of each panel. (A) PTB1, (B) reduced PTB1 (buffer supplemented with 5 mM DTT), (C) PTB1-1234, (D) PTB1-C23S.

the dimer peaks; it also resulted in the appearance of a peak at 5.7 kDa, most likely a minor proteolytic contaminant (Fig. 5B). The presence of dimers could not be restored with increasing pressure.

To confirm that these dimers were disulphide mediated, we performed ESI-MS analysis of the constructs PTB1-1234 and PTB1-C23S, in which Cys23 has been deleted and mutated, respectively. Only monomeric protein could be identified for PTB1-1234 ($53,108 \pm 5$ Da) and PTB1-C23S ($58,349 \pm 17$ Da) under both oxidizing (Fig. 5C,D) and reducing (data not shown) conditions. Together these data strongly indicate that the PTB1 dimers formed in oxidizing conditions are the result of covalent disulphide interactions between the cysteine residue at position 23 on each protein molecule.

DISCUSSION

This report provides new biophysical and biochemical evidence that PTB exists as a monomer in solution, confirming recent findings derived from analytical ultracentrifugation experiments with PTB (Simpson et al. 2004; Amir-Ahmady

et al. 2005). Moreover, in conjunction with results from NMR and small-angle X-ray scattering experiments (Simpson et al. 2004), the present observations provide an explanation for the previous misconception that PTB exists as a homodimer in solution. Firstly, the asymmetrical rod-shaped conformation of PTB1 leads to a significant overestimation of the molecular weight of PTB monomers when native gels or size-exclusion techniques are used. Secondly, we have shown that although PTB dimers can form under oxidizing conditions, these are entirely due to the formation of an intermolecular disulphide bridge involving Cys23 on both partners. We suggest that both of these factors contributed to the apparent observation of PTB dimerization in previous analyses of PTB oligomerization, which used gel filtration chromatography, *in vitro* pull-down assays, nondenaturing PAGE, and glutaraldehyde cross-linking, and were all performed under oxidizing conditions (Perez et al. 1997; Oh et al. 1998). In experiments performed under reducing conditions (Figs. 1, 2, 3, 5), PTB behaved as a monomer; we found no evidence for noncovalent dimerization.

In vitro pull-down assays (Perez et al. 1997) and glutaraldehyde cross-linking experiments (Oh et al. 1998) using recombinant PTB fragments lacking Cys23 provided evidence for self-association of PTB that cannot be attributed to the formation of disulphide bridges and led to the suggestion that RRM2 mediated noncovalent association of PTB monomers. However, in these studies, the self-association behavior was principally observed for constructs in which RRM2 was located either at the N or the C terminus of the truncated protein, an exposed position that is not found in full-length PTB. Subsequent analyses using both NMR and analytical ultracentrifugation have shown that these types of fragment aggregate nonspecifically (Simpson et al. 2004).

The formation of disulphide-mediated PTB dimers cannot account for self-association behavior deduced from yeast two-hybrid experiments (Oh et al. 1998), since the reducing environment of the yeast nucleus would have prevented disulphide bridge formation. However, it remains possible that the apparent PTB–PTB interaction observed by this technique could have been mediated by interactions with other proteins or with RNA. Yeast two-hybrid data need to be supported by more direct analyses of protein–protein interactions, but the balance of the data now suggests that PTB is indeed monomeric.

Until recently it has been widely accepted that human PTB exists as a homodimer in solution. It is now apparent that under reducing conditions PTB exists as a monomer and that this is the physiologically relevant state of the protein in the reducing environment of the cell. This study provides a revealing example of the care that needs to be taken in determining the oligomeric state of intracellular proteins. The findings will also help to clarify analyses of the likely stoichiometry of PTB–RNA complexes and the formulation of models of PTB function. Of course, it remains possible that multimers of PTB will form when

the protein associates with RNA targets or other proteins in vivo (Chou et al. 2000; Polydorides et al. 2000; Huttelmaier et al. 2001; Amir-Ahmady et al. 2005).

MATERIALS AND METHODS

Plasmid construction and protein expression

The plasmids PTB1 (residues 1–531) and PTB1-1234 (residues 55–531) expressing human PTB have been described previously (Simpson et al. 2004). Mutant constructs PTB1-C23S and PTB1-C250S/C251S were created by QuikChange site-directed mutagenesis of PTB1 using the following primer pairs, in which the mutated codons are underlined: C23S, GACGAGCTCTTCTCTA CTAGTGTCCTAACGGACC and GGTCCGTTAGTGACACTAGTAGAGAAGAGCTCGTC; C250S/C251S, AGAACATCTACAACGCCTCCTCCACGCTGCGCATC and GATGCGCAGCGTTGGAGGAGGCGTTGTAGATGTTCT. Mutations were confirmed by sequencing. Proteins were expressed essentially as described (Conte et al. 2000) by using SG13009 cells and were purified by using TALON (Clontech) affinity resin.

BN-PAGE

BN-PAGE analysis was performed based on the method of Schagger and von Jagow (1991) using 10 μ g of protein in 20 mM Tris (pH 7.7), 200 mM NaCl, and 10% glycerol. To create a reducing environment, this was supplemented with 10 mM DTT. Electrophoresis was performed by using prefocused (100 V, 60 min, 4°C) 4%–15% Tris-HCl gradient gels (Biorad) for 2–4 h at 15 mA and 4°C. The cathode buffer was 50 mM Tricine, 15 mM BisTris-HCl (pH 7.0), and 0.02% (w/v) Coomassie blue G; the anode buffer, 50 mM BisTris-HCl (pH 7.0). To enhance final visualization of protein bands, gels were completely destained (40% EtOH, 10% acetic acid) and then restained (50% EtOH, 10% acetic acid, 0.1% [w/v] Coomassie blue). A linear plot of M_r against distance migrated was obtained for each gel by using the following proteins: β -amylase (200 kDa), alcohol dehydrogenase and its dissociated subunits (150 kDa and 50 kDa), HSA monomer and disulphide-linked dimer (66 kDa and 132 kDa), and chicken albumin (45 kDa).

SDS-PAGE

One microliter of 10 μ M protein stocks was subjected to standard electrophoresis in 12% SDS-polyacrylamide gels using both reducing and nonreducing sample loading buffers. Two times reducing sample loading buffer is 125 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 0.02% Bromophenol blue, and 730 mM β -mercaptoethanol. Oxidizing sample loading buffer did not contain β -mercaptoethanol. Samples were not boiled prior to electrophoresis.

Size-exclusion chromatography

Size-exclusion chromatography was performed as described previously (Simpson et al. 2004) by using either a reducing (25 mM Tris at pH 7.2, 250 mM NaCl, 2 mM DTT), or a nonreducing (25 mM Tris at pH 7.2, 250 mM NaCl) buffer.

NMR spectroscopy

Two-dimensional ^1H - ^{15}N NMR spectra were recorded at 303 K on a 500-MHz four-channel Bruker DRX500 spectrometer equipped with a z-shielded gradient triple resonance cryoprobe. Samples of reduced ^{15}N -labeled PTB1 RRM2 were $\sim$ 100–200 μ M (pH 6.5) in 50 mM Na phosphate buffer, 150 mM NaCl, 10 mM DTT, and 1 mM NaN_3 . Spectra of oxidized protein were acquired both by allowing samples containing DTT to oxidize in air and by preparing fresh protein in identical buffer but with the omission of DTT. Chemical shift changes upon oxidation were judged significant by using a weighted sum of $10(\Delta\delta^{\text{H}}) + \Delta\delta^{\text{N}}$ (ppm) as described previously (Simpson et al. 1999).

Time-of-flight electrospray ionization MS

For analysis under native conditions, purified protein was buffer exchanged into 50 mM ammonium acetate (pH 6.5) and diluted to a final concentration of 10 μ M in 50 mM ammonium acetate (pH 6.5). Spectra were also recorded on samples supplemented with 2–5 mM DTT. Data were acquired by using a modified QToF2 instrument (Waters) fitted with a standard Z-spray nano-flow electrospray source. Sample solutions were sprayed from borosilicate glass capillaries (1 mm outer diameter $\times$ 0.5 mm inner diameter) that had been drawn down to a closed taper using a Model P-97 Flaming/Brown micropipette puller (Sutter Instrument) and coated with gold using a SEM coater (Polaron). The tapered end of the capillary was cut under a microscope. A low backing pressure of nitrogen gas was used to initiate and maintain flow through the capillary. All data were acquired in positive ion mode using a nitrogen cone gas flow to aid desolvation. Throttling of the source rotary pump was used to control instrument pressures for collisional cooling. Spectra were acquired with the following parameters: capillary voltage, 1.8 kV; cone voltage, 200 V; extractor voltage, 0 V; 1×10^{-2} mbar indicated source pressure; and 6.9×10^{-7} mbar indicated ToF analyser pressure. All data were processed by using MassLynx software, and cesium iodide solution was employed for calibration.

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