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Segmental isotope-labeling of the intrinsically disordered protein PQBP1

Yuko Nabeshima [a,b], Mineyuki Mizuguchi [a,b,*], Asagi Kajiyama [a], Hitoshi Okazawa [c,d]

[a] Faculty of Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
[b] Graduate School of Innovative Life Science, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
[c] Department of Neuropathology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
[d] Center for Brain Integration Research, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

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ABSTRACT

Polyglutamine tract-binding protein 1 (PQBP1) is an intrinsically disordered protein abundantly expressed in the brain. Mutations in the *PQBP1* gene are causative for X-linked mental retardation disorders. Here, we investigated the structure of the C-terminal segment within the context of full-length PQBP1. We produced a segmentally isotope-labeled PQBP1 composed of a non-labeled segment (residues 1–219; N-segment) and a $^{13}C/^{15}N$-labeled segment (residues 220–265; C-segment). Our results demonstrate that the segmental isotope-labeling combined with NMR spectroscopy is useful for detecting a very weak intra-molecular interaction in an intrinsically disordered protein.
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1. Introduction

The intrinsically disordered proteins (IDPs) are a group of proteins without a rigid structure that comprise a large part of the proteome [1–4]. IDPs play an important role for biological functions such as transcription, translation, signal transduction, molecular chaperoning, and cell-cycle regulation [1–4]. Polyglutamine tract-binding protein 1 (PQBP1) is an intrinsically disordered protein abundantly expressed in the brain [5–7]. PQBP1 is composed of 265 amino acid residues and contains a WW domain (WWD), a polar amino acid-rich domain (PRD), and a C-terminal domain (CTD). The functions of PQBP1 include the regulation of transcription and pre-mRNA splicing [7]. The WWD of PQBP1 interacts with RNA polymerase II [7] and the splicing factor WBP11/NpwBP/SIPP1 [8,9], while CTD interacts with the spliceosomal protein U5-15kD [10,11]. However, the structural characteristics of PQBP1, which are important for understanding its functions, are not fully understood.

We have previously shown that PQBP1 is composed of a small folded WWD and a long disordered region longer than 180 residues: its PRD and CTD are disordered under physiological conditions [12,13]. In our previous study, we analyzed the conformations of an isolated fragment corresponding to residues 223–265 of PQBP1. The 223–265 fragment of PQBP1 is highly disordered in the free state and includes the YxxPxxVL motif, which is essential for the interaction with the spliceosomal protein U5-15kD [13,14]. However, the isolated fragment may not represent all structural characteristics of the corresponding segment within the full-length protein. Therefore, we investigated the structure of the 223–265 segment within the context of full-length PQBP1.

It is difficult to investigate the conformation of a long disordered segment within the context of an intact protein by nuclear magnetic resonance (NMR) spectroscopy. This difficulty is mainly due to the severe overlap of resonances arising from residues located in the disordered segment [15,16]. To overcome this difficulty, we produced a segmentally isotope-labeled PQBP1 using the expressed protein ligation (EPL) method (Fig. 1a). EPL is based on native chemical ligation in which a fragment with an N-terminal cysteine is chemically ligated to another fragment with a C-terminal thioester

Abbreviations: CTD, C-terminal domain; EPL, expressed protein ligation; HPLC, high-performance liquid chromatography; IDP, intrinsically disordered protein; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass analysis; NMR, nuclear magnetic resonance; PRD, polar amino acid-rich domain; PQBP1, polyglutamine tract-binding protein 1; N-fragment, PQBP1(1–219) with a C-terminal thioester group; C-fragment, PQBP1(220–265) with a Gly220Cys mutation; N-segment, residues 1–219 of PQBP1; C-segment, residues 220–265 of PQBP1; MESNA, sodium 2-sulfanylethanesulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WWD, WW domain

* Corresponding author at: Faculty of Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. Fax: +81 76 434 5061.
E-mail address: mineyuki@pha.u-toyama.ac.jp (M. Mizuguchi).

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group [17–20]. Our results demonstrate that the segmental isotope-labeling combined with NMR spectroscopy is useful for detecting a very weak intra-molecular interaction in an intrinsically disordered protein.

2. Materials and methods

In order to obtain PQBP1(1–219) with a C-terminal thioester group (N-fragment), we used MxeGyrA intein and a chitin-binding domain (CBD) [18,19]. The DNA encoding the fusion protein PQBP1(1–219)-intein-CBD was inserted into a cold-shock plasmid, pCold IV (Takara Bio). The fusion proteins were expressed in *Escherichia coli* C41(DE3) harboring the pCold IV plasmid. Protein expressions were induced by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM, when the OD_{600nm} of the cell culture reached 0.4–0.5. After 24-h cultivation at 15 °C, the cells were harvested by centrifugation. The cells were resuspended in 20 mM HEPES and 500 mM NaCl (pH 8.0) and lysed by sonication on ice. The fusion protein of PQBP1(1–219)-intein-CBD was detected in a soluble fraction. A solution containing the fusion protein was applied to a chitin column equilibrated with 20 mM HEPES and 500 mM NaCl (pH 8.0). After the column was washed with the same buffer, the suspension of chitin beads was transferred to a 50 mL tube, and sodium 2-sulfanylethanesulfonate (MESNA) was added to a final concentration of 50 mM. The suspension of chitin beads was incubated at 37 °C overnight, then transferred to an empty column where the flow-through was collected by adding 20 mM HEPES, 500 mM NaCl, and 50 mM MESNA (pH 8.0).

In order to obtain PQBP1(220–265) with a Gly220Cys mutation (C-fragment), we used SSpDnaB intein and CBD [19]. The fusion protein of CBD-intein-PQBP1(220–265) was expressed in *E. coli* BL21(DE3) with a cold-shock vector, pCold IV. To isotopically label PQBP1(220–265) with ^{13}C and ^{15}N, we used $^{13}C/^{15}N$-labeled C.H.L. medium (Chlorella Industry). Protein expressions were induced by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM, when the OD_{600nm} of the cell culture reached 0.4–0.5. After 24-h cultivation at 15 °C, the cells were harvested by centrifugation and resuspended in 200 mM Tris–HCl and 500 mM NaCl (pH 8.5 at 20 °C). After cell lysis by sonication, a soluble fraction containing the fusion protein was applied to a chitin column equilibrated with 20 mM Tris–HCl and 500 mM NaCl (pH 8.5 at 20 °C). After the column was washed with the same buffer, a suspension of chitin beads was transferred to a 50 mL tube. Dithiothreitol was added to a final concentration of 100 mM, and the pH was adjusted to 7.6–8.0 at 25 °C by adding 1 M HCl. The suspension of chitin beads was incubated at 37 °C overnight. The suspension was transferred to an empty column, and the flow-through was collected by adding 20 mM Tris–HCl, 500 mM NaCl, and 1 mM dithiothreitol (pH 7.5 at 25 °C). The C-fragment was further purified by reverse-phase high-performance liquid chromatography (HPLC) with a COSMOSIL 5C18-AR300 column (Nacalai Tesque, Japan). Fractions containing the C-fragment were collected and dialyzed against 20 mM Tris–HCl, 100 mM NaCl, and 1 mM dithiothreitol (pH 8.5 at 25 °C). After dialysis, dithiothreitol was added to a final concentration of 10 mM. The solution was concentrated using an Amicon Ultra centrifugal filter device (3.5 kDa cutoff; Millipore), followed by dialysis against 20 mM HEPES, 100 mM NaCl, and 5 mM MESNA (pH 8.0).

The protein ligation reaction was performed according to the following procedure. The N-fragment was mixed with the C-fragment at a 1:10 M ratio (N-fragment:C-fragment). The concentrations of the N- and C-fragments in the mixture were 0.24 mM and 2.2 mM, respectively. The mixture solution was dialyzed against 20 mM HEPES, 100 mM NaCl, and 5 mM MESNA (pH 8.0) at 37 °C for 16 h. After dialysis, dithiothreitol was added to the solution, to a final concentration of 10 mM. The solution was incubated at 37 °C for 2 h and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The segmentally isotope-labeled protein was separated from unreacted fragments by reverse-phase HPLC with a COSMOSIL Protein-R column (Nacalai Tesque, Japan). Matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF MS) analysis showed that the segmentally isotope-labeled PQBP1 and PQBP1(1–219) have no methionine at the N-terminus. MALDI-TOF MS analysis was performed on a Bruker Daltonics autoflex-T1 mass spectrometer. The mass spectrometer was calibrated with Protein Standard II (Bruker Daltonics), based on signals from trypsinogen (m/z 23 982) and protein A (m/z 22 307 and 44 613).

The DNA encoding PQBP1(1–219) was also inserted into the pOPTH plasmid. PQBP1(1–219) with an N-terminal His-tag (MAHHHHHH) was expressed in *E. coli* C43(DE3) harboring the pOPTH plasmid. Purification of PQBP1(1–219) was done with an Ni–NTA agarose resin (QIAGEN), followed by further purification by reverse-phase HPLC with a COSMOSIL 5C18-AR300 column (Nacalai Tesque, Japan). MALDI-TOF MS analysis showed that His-tagged PQBP1(1–219) has no methionine at the N-terminus. Full-length PQBP1 was obtained in the same manner as the His-tagged PQBP1(1–219). The full-length $^2H/^{15}N$-labeled PQBP1 was produced using M9 medium containing ^{15}N-NH_4Cl and ^2H_2O [21].

NMR experiments were performed on an 800 MHz Bruker Avance spectrometer equipped with a cryoprobe. NMR samples contained 20 mM sodium phosphate (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 10% D_2O, 1 mM NaN_3 and 20 μM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt except where stated otherwise. Backbone resonances were assigned sequence-specifically by analyzing conventional three-dimensional triple resonance spectra including CBCANH [22], CBCA(CO)NH [23], HNCO [24], HN(CA)CO [25], HNCA [24], and HN(CO)CA [24]. NMR data were processed with NMRPipe [26] and analyzed with NMRView [27]. The ^1H chemical shifts were directly referenced to the resonance of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt, while the ^{13}C and ^{15}N chemical shifts were indirectly referenced with the absolute frequency ratios [28].

3. Results

3.1. Segmental isotope-labeling of PQBP1

We utilized EPL to perform selective isotope-labeling of a C-terminal 46-residue segment within full-length PQBP1 (Fig. 1a). The N- and C-fragment correspond to residues 1–219 and residues 220–265 of PQBP1, respectively. The C-terminus of the N-fragment is thioesterified, which is essential for the protein ligation [17–20]. The N-terminal Gly of the C-fragment is mutated to Cys, which is required for EPL (Fig. 1a). The N-fragment is non-labeled, while the C-fragment is $^{13}C/^{15}N$-labeled, and thus the residues 220–265 (C-segment) are only labeled with ^{13}C and ^{15}N within the full-length PQBP1. The N- and C-fragments were expressed as a fusion protein with MxeGyrA intein and SSpDnaB intein, respectively [18,19] (Supplementary Figs. 1 and 2). The N-fragment was fused to the N-terminus of the MxeGyrA intein, while the C-fragment was fused to the C-terminus of the SSpDnaB intein. Cleavage between the N-fragment and MxeGyrA intein was done by adding MESNA, which generated a C-terminal thioester group of the N-fragment [18,19] (Supplementary Fig. 1). It is known that the nature of the C-terminal thioester influences the efficiency of protein ligation, and that the C-terminal thioester generated by MESNA is efficient for EPL [18]. Cleavage between the C-fragment and SSpDnaB intein was induced at pH 7.6–8.0

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