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The Neuroendocrine Protein 7B2 Is Intrinsically Disordered

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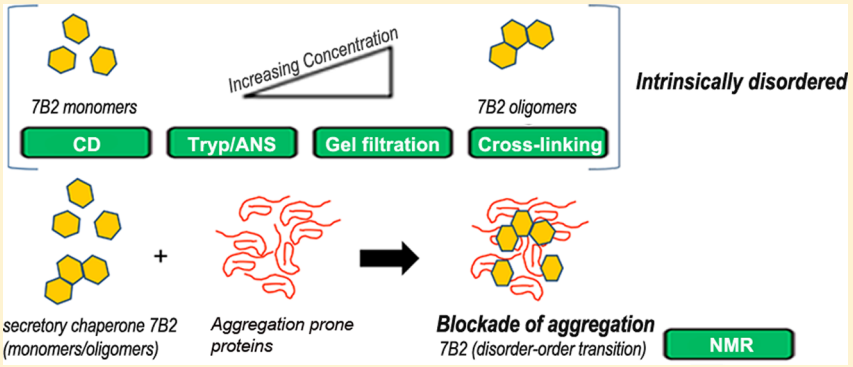

ABSTRACT: The small neuroendocrine protein 7B2 has been shown to be required for the productive maturation of proprotein convertase 2 (proPC2) to an active enzyme form; this action is accomplished via its ability to block aggregation of proPC2 into nonactivatable forms. Recent data show that 7B2 can also act as a postfolding chaperone to block the aggregation of a number of other proteins, for example, α-synuclein. To gain insight into the mechanism of action of 7B2 in blocking protein aggregation, we performed structural studies of this protein using gel filtration chromatography, intrinsic tryptophan fluorescence, 1-anilino-8-naphthalenesulfonate (ANS) binding, circular dichroism (CD), and nuclear magnetic resonance (NMR) spectroscopy. Gel filtration studies indicated that 7B2 exists as an extended monomer, eluting at a molecular mass higher than that expected for a globular protein of similar size. However, chemical cross-linking showed that 7B2 exhibits concentration-dependent oligomerization. CD experiments showed that both full-length 27 kDa 7B2 and the C-terminally truncated 21 kDa form lack appreciable secondary structure, although the longer protein exhibited more structural content than the latter, as demonstrated by intrinsic and ANS fluorescence studies. NMR spectra confirmed the lack of structure in native 7B2, but a disorder-to-order transition was observed upon incubation with one of its client proteins, α-synuclein. We conclude that 7B2 is a natively disordered protein whose function as an antiaggregant chaperone is likely facilitated by its lack of appreciable secondary structure and tendency to form oligomers.

7B2 is a small secretory protein that is known to be required for the productive maturation of the subtilisin-like endoprotease prohormone convertase 2 (proPC2).[1−3] Extensive work by our group and other groups has established the motifs and domains required for the interaction between 7B2 and proPC2.[4−9] The 7B2 protein itself is cleaved, most likely by a furin-like enzyme, to yield the 21 kDa amino-terminal domain and a 31-residue carboxy-terminal fragment.[10,11] The C-terminal domain, also known as the CT peptide, is a potent inhibitor of PC2,[12] whereas the N-terminal domain is essential for the effective maturation of proPC2 within the secretory pathway.

Apart from its role in transporting folded proPC2 to the Golgi apparatus in neuroendocrine cells (reviewed in ref 13), 7B2 has been shown to have antiaggregant properties,[14,15] blocking oligomer formation and aggregation of both proPC2[15] and insulin-like growth factor 1 (IGF1).[14] We have recently shown that co-incubation of neurodegeneration-related peptides and proteins, such as β-amyloid, α-synuclein, and tau, with micromolar quantities of 7B2 results in inhibition of fibril formation (Helwig et al., manuscript submitted for publication). Recent proteomics studies have provided evidence that the 7B2 protein may be a potential biomarker for early detection of Alzheimer's and Parkinson's disease.[16−19] These results, coupled with the selective expression of 7B2 in endocrine, neural, and neuroendocrine cells,[20] led to the hypothesis that this protein might be a member of a novel family of secretory chaperones that prevent the self-association of proteins with tendencies toward aggregation, such as neurodegenerative-related proteins (Helwig et al., manuscript submitted for publication).

To date, nothing is known about the three-dimensional structure of this 186-residue protein. The minimal domain necessary for the biological function of 7B2 with proPC2 is a 36-residue internal peptide that consists of a proline-rich

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segment, a putative α helix, and a disulfide bridge.[9] Mutation of the fourth proline residue in the PDPPNPCP stretch (residues 88−95; numbering does not include the signal peptide) disrupts the ability of the protein to facilitate the maturation of proPC2.[6] The structural motifs responsible for inhibition of amyloid aggregation are not yet known though clearly involve the N-terminal domain (Helwig et al., manuscript submitted for publication).

All vertebrate 7B2 sequences are extremely well conserved, with almost 97% sequence identity. However, the level of sequence identity between vertebrate 7B2 and the *Caenorhabditis elegans* protein is low, only 20−23% overall. An interesting feature of the *C. elegans* 7B2 sequence is the conservation of the signature proline-rich motif, PPNPCP, which has been identified as a critical domain for facilitation of proPC2 maturation.[6] It is not clear whether the large differences in the amino acid sequence of *C. elegans* 7B2 alter its secondary structure as compared to vertebrate 7B2s. Despite the low level of overall sequence conservation, the physiological functions of the *C. elegans* protein with regard to proPC2 appear to be similar to those of the vertebrate proteins.[21]

To gain a better understanding of structural contributions to the physiological functions of the full-length and truncated forms of the 7B2 protein, we examined potential differences in folding states between each of these variants, shown in Figure 1,

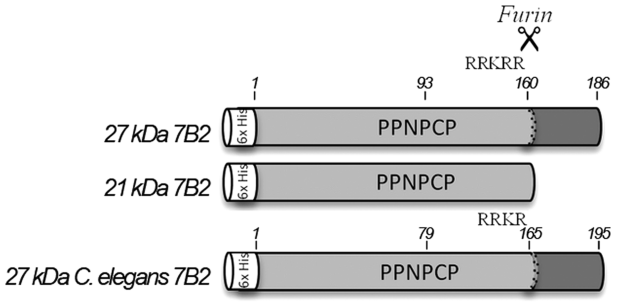

Figure 1. Domain structures for the proteins used in our study.

using intrinsic tryptophan fluorescence, 1-anilino-8-naphthalenesulfonate (ANS) binding, circular dichroism, and nuclear magnetic resonance (NMR) spectroscopy. The hydrodynamic properties of 21 kDa rat 7B2 were also analyzed using gel filtration chromatography. This was followed by primary structural analysis of *C. elegans* and rat 27 kDa 7B2s using the disorder prediction algorithm PONDR VL-XT.[22] Our data indicate that 7B2 is an intrinsically disordered protein with a tendency toward oligomerization at high protein concentrations.

METHODS

Expression and Purification of 7B2 Variants. Rat and *C. elegans* 7B2 variants previously cloned into the pQE30 vector[6,21,23] were subcloned into the pET45b(+) vector with an attached N-terminal hexahistidine tag. The vectors containing the different 7B2 constructs, rat 27 kDa, rat 21 kDa, and *C. elegans* 27 kDa, were transformed into the Bl21(DE3) strain of *Escherichia coli* (GE Healthcare). Expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested by centrifugation after being induced for 4 h, pelleted, and stored at −80 °C. The pellets were thawed and lysed using BugBuster Protein Extraction Reagent (Novagen) containing protease inhibitors (*Complete* EDTA-free, Roche). The lysed cells were centrifuged for 50 min at 13000 rpm. The supernatant was loaded onto a 5 mL HisTrap HP column (GE Healthcare), equilibrated with 20 mM Tris-HCl, 0.1 M NaCl, and 5 mM imidazole (pH 7.9). The column was washed with 5 volumes of the same buffer containing 500 mM NaCl and eluted with 175 mM imidazole. The protein was diluted five times in 20 mM bis-Tris, 2 mM $CaCl_2$, and 0.4 mM octyl glucoside (pH 6.5) and loaded onto a 1 mL MonoQ column (GE Healthcare). The column was washed and eluted with 10 volumes of the same buffers containing first 100 mM NaCl and then 500 mM NaCl. The eluted fractions were desalted into the final buffer with a HiPrep 26/10 column (GE Healthcare) and concentrated using an Amicon Ultra 30K spin concentrator (Millipore). The purity and integrity of the 7B2 variants were assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and matrix-assisted laser desorption mass spectroscopy (MALDI-TOF MS) at the University of Maryland, Baltimore, mass spectrometry core facility. The theoretical molecular masses for recombinant 7B2 and its derivatives are 21901 Da (27 kDa WT), 17611 Da (21 kDa WT), and 23093 Da (27 kDa *C. elegans*). These compare well with the mass spectroscopic experimentally derived molecular masses of 21921.6 and 17674.4 Da for the 27 and 21 kDa 7B2 proteins, respectively.

To obtain uniformly ^{15}N-labeled protein samples, the 7B2 expression vectors were grown in minimal medium supplemented with 0.6 g/L $^{15}NH_4Cl$ (Cambridge Isotope Laboratories) and purified using the procedures described above.

The eukaryotic 21 kDa 7B2 protein was purified from CHO cells stably overexpressing 7B2. Conditioned serum-free OptiMem medium from CHO cells was diluted 2.5 times in 20 mM bis-Tris, 2 mM $CaCl_2$, and 0.4 mM octyl glucoside (pH 6.5) and loaded onto a DEAE column (GE Healthcare). The column was washed with the with 7 and 12 % of the same buffer containing 1 M sodium acetate. The fractions that were eluted with 200 mM sodium acetate were subsequently diluted 10 times and subjected to Mono Q chromatography, HiPrep 26/10 desalting, and gel filtration following the same procedures described above for the purification of the bacterial recombinant 7B2 protein.

Size Exclusion Chromatography. One hundred microliters of 21 kDa 7B2 at a concentration of either 0.5 or 2 mg/mL was injected onto a Superdex 75 10/300 GL column connected to a Superdex 200 10/300GL (GE Healthcare) column, previously equilibrated with 20 mM bis-Tris, 150 mM NaCl, 2 mM $CaCl_2$, and 0.4 mM octyl glucoside (pH 6.5). The gel filtration column was calibrated using Bio-Rad standards, including thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobulin (17 kDa), and vitamin B_{12} (1.35 kDa). The elution volumes of the standards were used to determine the apparent molecular mass of 7B2. The gel filtration experiments were conducted with two independent recombinant 7B2 preparations.

In Vitro Cross-Linking. The 7B2 gel filtration peaks were treated with 0.1% glutaraldehyde for 30 min at 4 °C. The reactions were quenched by the addition of 1 M Tris for 15 min at room temperature. The samples were then analyzed by SDS−PAGE (4 to 20% gradient). The 27 and 21 kDa 7B2 proteins were also cross-linked with 0.1% glutaraldehyde at concentrations ranging from 30 to 7.5 μM for 30 min at 4 °C. Samples were separated on a 4 to 20% gradient SDS−PAGE gel.[3] The images acquired by scanning of the SDS−PAGE gel

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were digitized using UN-SCAN-IT version 6.1 (Silk Scientific Inc.).

Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF MS). 7B2 samples were mixed in a 1:1 (v/v) ratio with a sinapinic acid matrix (Thermo-Scientific). Aliquots of 1 μL were spotted on the MALDI-TOF plate using the dried droplet method, and mass spectra were recorded in a Bruker Autoflex mass spectrometer operated in linear mode. The instrument was calibrated using a protein mixture ranging from 4000 to 20000 Da (protein calibration standard I, Bruker Daltonics).

Tryptophan Fluorescence. Fluorescence spectra were recorded on a SpectraMax M2e fluorescence spectrophotometer (Molecular Devices) at 25 °C. The concentration of samples was 2 μM in a buffer consisting of 20 mM bis-Tris, 2 mM $CaCl_2$, 150 mM NaCl, and 0.4 mM octyl glucoside at pH 6.5. The protein samples were incubated in buffers containing 0, 1, 2, 3, 5, 7, or 9 M urea for 18 h at 4 °C before the fluorescence was measured. The excitation wavelength was set to 295 nm, and emission spectra were recorded between 300 and 400 nm at 1 nm intervals.

1-Anilino-8-naphthalenesulfonate (ANS) Binding. The fluorescent probe molecule 1-anilino-8-naphthalenesulfonate (ANS) was obtained from Sigma (Sigma-Aldrich). ANS, at a concentration of 40 μM, was incubated with urea-denatured protein samples (as described in the previous section) for 1 h at 25 °C. ANS fluorescence spectra were recorded using a SpectraMax M2e fluorescence spectrophotometer at 25 °C. ANS was excited at 360 nm, and the emission spectra were recorded between 400 and 600 nm.

Circular Dichroism Spectroscopy. All purified protein samples were buffer-exchanged with 10 mM sodium phosphate buffer (pH 6.5), containing 0.4 mM octyl glucoside, using a HiTrap desalting column (GE Healthcare). The final concentration of all protein samples used for CD measurements was 20 μM. The precise concentrations for CD samples were determined using molar extinction coefficients, based on the number of tryptophan and tyrosine residues. CD spectra were recorded on a Jasco CD spectrophotometer. The spectra were recorded between 190 and 260 nm at intervals of 1 nm/s. The final spectra represent an average of three runs. The raw data (in millidegrees) were converted to mean residue ellipticity (MRE), expressed in θ degrees square centimeter per decimole using the following equation[24,25]

$$[\theta] = \frac{\theta \times 100M}{Cln} \quad (1)$$

where θ is the observed ellipticity in millidegrees, M is the molecular mass of the sample, C is the concentration in milligrams per milliliter, l is the optical path length in centimeters, and n is the number of residues.

The secondary structure was estimated using the K2D2 analysis tool (http://www.ogic.ca/projects/k2d2/index.html).[26]

NMR Spectroscopy. All NMR spectra were collected with a Bruker AVANCE NMR spectrophotometer (800.21 MHz for protons) equipped with four frequency channels and 5 mm triple-resonance z-axis gradient cryogenic probeheads. Protein samples were dissolved in a buffer composed of 20 mM bis-Tris, 2 mM $CaCl_2$, 0.4 mM octyl glucoside, and 150 mM NaCl (pH 6.5) to a final concentration of 0.5 mM. D_2O (10%) was added to lock the signal. Two-dimensional (2D) $^1H-^{15}N$ HSQC spectra were acquired at 283 K with a spectral width of 16 ppm and 32 scans. The 2D $^1H-^{13}C$ natural abundance carbon HSQC spectrum was also acquired at 283 K with 256 scans.

^{15}N-labeled 27 kDa 7B2 protein was incubated with human α-synuclein (kindly supplied by N. Lorenzen and D. E. Otzen) at a ratio of 1:2 in the same buffer that was used for 7B2 alone. 2D $^1H-^{15}N$ TROSY spectra were acquired at 283 K with a spectral width of 16 ppm and 200 scans.

RESULTS

Gel Filtration Chromatography and in Vitro Cross-Linking. The hydrodynamic characteristics of 21 kDa 7B2 were analyzed by size exclusion chromatography using a Superdex 75 10/300GL column connected to a Superdex 200 10/300GL column. A single peak was observed in the elution profile with an apparent molecular mass of ~42000 Da (Figure 2a), which is larger than that of the molecular mass obtained by

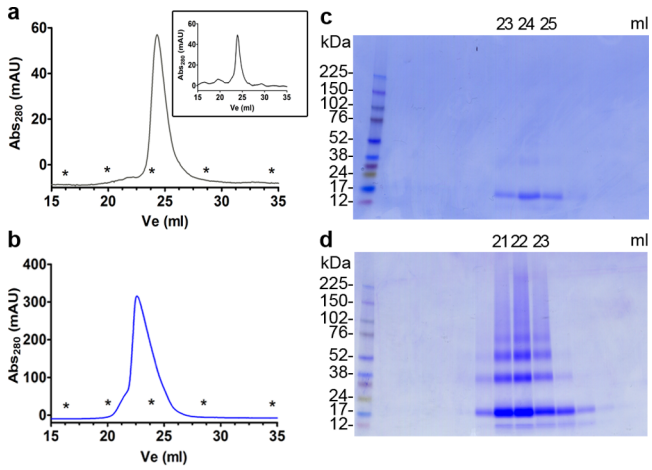

Figure 2. Size exclusion chromatography and cross-linking. (a and b) Size exclusion chromatogram of 21 kDa 7B2 at 0.5 and 2 mg/mL (blue line), respectively. The inset of panel a shows the size exclusion chromatogram of eukaryotic 21 kDa 7B2 at 0.5 mg/mL. The molecular mass standards, thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobulin (17 kDa), and vitamin B_{12} (1.35 kDa), are shown with asterisks. (c and d) Chemical cross-linking (0.1% glutaraldehyde) of gel filtration experiments using 0.5 and 2 mg/mL 21 kDa 7B2, respectively.

mass spectrometry (17674.4 Da). This result suggests either that 7B2 forms a dimer, as previously suggested,[27] or that it exists as an extended monomer. The elution volume for the 7B2 peak was reproducibly shifted by 1.8 mL, corresponding to an upward molecular mass shift of ~20 kDa, when a higher concentration of the protein was applied to the gel filtration column. The chromatography fractions were subsequently cross-linked using glutaraldehyde (panels c and d of Figure 2, low- and high- concentration gel filtration chromatographies, respectively) and analyzed by SDS–PAGE. A major band that corresponds to the monomer mass was observed; dimeric, trimeric, and higher-order oligomers were also present, but at lower concentrations. We also tested whether the presence of the His tag in the recombinant 7B2 protein affected the elution profile. Eukaryotic 21 kDa 7B2 was purified from the medium of CHO cells overexpressing the native rat protein and analyzed by size exclusion as described above. The results, presented in Figure 2a (inset), show that this form of 7B2 also eluted at ~24 mL, i.e., with an apparent molecular mass much greater than

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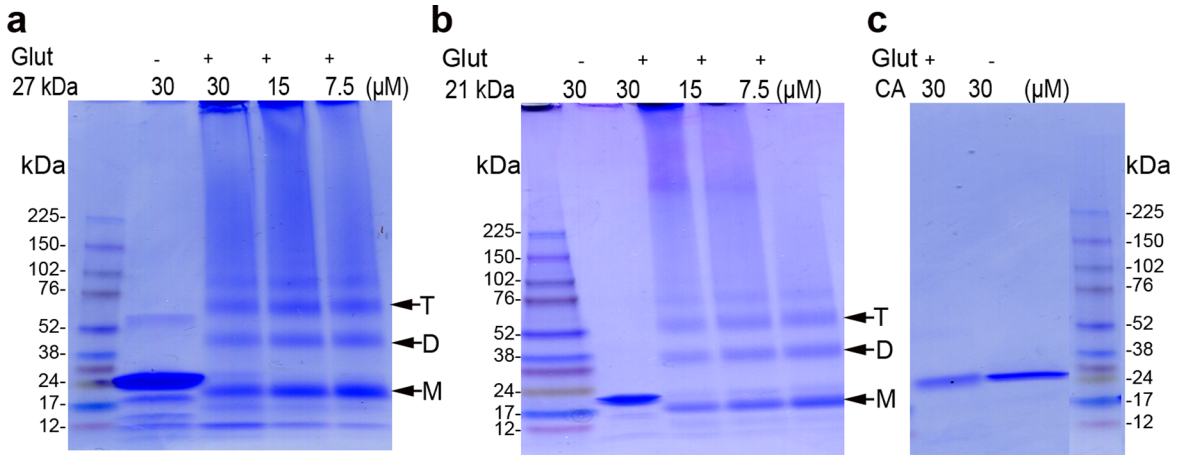

Figure 3. Chemical cross-linking of 27 and 21 kDa 7B2s: concentration dependence of multimer formation. (a) The 27 kDa 7B2 was cross-linked with 0.1% glutaraldehyde at final concentrations ranging from 7.5 to 30 μM. (b) The 21 kDa 7B2 was cross-linked with 0.1% glutaraldehyde at final concentrations ranging from 7.5 to 30 μM. (c) Carbonic anhydrase, at 30 μM, cross-linked with 0.1% glutaraldehyde. Bands corresponding to monomer (M), dimer (D), and trimer (T) are marked on the gels with arrows.

that of a monomer. We conclude that the aberrant molecular mass is not a function of the His tag.

We also tested if dilution played a role in determining whether 7B2 exists as a monomer or an oligomer. 7B2 samples were cross-linked at varying protein concentrations using a fixed amount of glutaraldehyde. These data, shown in panels a and b of Figure 3, demonstrate that both the 27 and 21 kDa forms of the 7B2 protein are prone to forming dimers, trimers, tetramers, and higher-order oligomers in a concentration-dependent manner. To visualize the ratio between monomers and oligomeric species at different protein concentrations, samples were analyzed by SDS−PAGE, loading equal mass for all concentrations. Cross-linking at 30 μM resulted in major oligomerization of both the 27 and 21 kDa forms (Figure 3a,b, lane 2). The extent of oligomerization was gradually reduced with decreasing concentrations of 7B2. No such behavior was observed for the similarly sized globular protein carbonic anhydrase (Figure 3c). Glutaraldehyde cross-linking is known to affect the migration of unstructured proteins on SDS−PAGE by reduction of the hydrodynamic radius and increased level of SDS binding.[28] This likely explains the aberrant molecular mass of the monomer, dimer, and trimer bands following cross-linking.

The homogeneity of the 7B2 protein preparations was ascertained by digitization of the lanes corresponding to the non-cross-linked 27 and 21 kDa proteins in panels a and b of Figure 3 using Un-Scan-IT, as described by the manufacturer. This quantitation indicates that 99.9% of the total pixel intensity of protein in each lane corresponds to the 27 and 21 kDa proteins.

Secondary Structure Analysis Using Intrinsic Tryptophan Fluorescence and ANS Fluorescence. The 21 kDa N-terminal and 31-residue C-terminal domains of the 7B2 protein (which together constitute the 27 kDa protein) are responsible for two very different functions with regard to proPC2, inhibition of aggregation and potent inhibition of enzyme activity, respectively.[12] To understand the contribution of the C-terminal domain to the overall structure of the protein, we compared the intrinsic tryptophan fluorescence, ANS binding, and circular dichroism spectra of the full-length protein with those of the 21 kDa protein.

7B2 samples, at a concentration of 2 μM, were exposed to increasing concentrations of urea (0−9 M), and we measured fluorescence by exciting the samples at 295 nm and recording the emission spectra between 300 and 400 nm. The results are shown in Figure 4. The 27 kDa rat protein (Figure 4a) has a

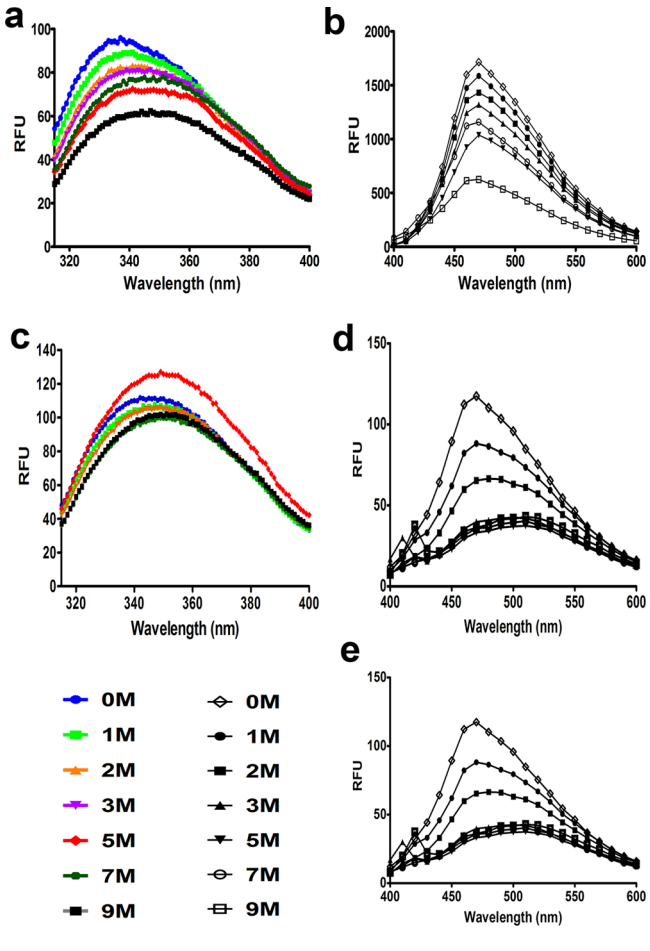

Figure 4. Tryptophan (left) and ANS (right) fluorescence spectra of 7B2 variants. (a and b) Wild-type rat 27 kDa 7B2. (c and d) Wild-type rat 21 kDa 7B2. (e) *C. elegans* 27 kDa 7B2.

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single tryptophan residue at position 137. The fluorescence spectrum of the 27 kDa protein, in the native state, showed an emission maximum at 335 nm that was shifted to 351 nm upon the addition of 9 M urea as a denaturing agent. The shift indicates that the tryptophan residue is shielded from the aqueous phase in the native state and gradually becomes exposed to solvent by urea-induced protein unfolding, emitting at 351 nm, the wavelength typical for L-tryptophan in water.[29] Urea-induced unfolding was also studied using ANS binding, as ANS fluorescence is known to be highly environmentally sensitive. The fluorescence intensity of ANS is very low in polar environments, whereas binding of ANS to hydrophobic surfaces causes an increase in quantum yield (fluorescence intensity) coupled with a blue shift in the maximal emission wavelength.[30] ANS bound strongly to the 27 kDa 7B2 protein in the native state (0 M urea), as observed by a large increase in fluorescence intensity. However, upon exposure to increasing concentrations of urea, the ANS fluorescence intensity was linearly reduced, suggesting gradual unfolding of the 27 kDa 7B2 protein upon urea denaturation (Figure 4b).

In contrast, 21 kDa 7B2 exhibited an emission spectrum typical of proteins with exposed tryptophan residues with a maximum at 345 nm. This form of 7B2 was also much more vulnerable to urea-induced denaturation than the full-length protein. Exposure to only 2 M urea caused a red shift in the fluorescence maximum to 351 nm, as shown in Figure 4c. The ANS binding data for this form of 7B2 were very interesting as well. Along with a 44% reduction in ANS fluorescence intensity between 1 and 2 M urea, the emission maximum shifted to 510 nm (Figure 4d). This spectrum is very different from that of the 27 kDa protein, where no change in the ANS emission maximum, 470 nm, was observed even at the highest urea concentration of 5 M. These data indicate that the C-terminal fragment of the 7B2 protein greatly contributes to the maintenance of the tertiary structure of the protein.

The 27 kDa *C. elegans* 7B2 protein, which has only a single tryptophan residue located near the carboxy terminus, was not analyzed using the intrinsic tryptophan fluorescence technique as the tryptophan residue is most likely already solvent-exposed because of its location. The ANS binding data for this protein, depicted in Figure 4e, are also nonlinear, with maximal emission at 510 nm. This maximum is characteristic of unbound ANS and contrasts with the 470 nm emission maximum seen using the vertebrate 27 kDa 7B2 protein. These data reflect a possible absence of the hydrophobic clusters necessary for binding of ANS in *C. elegans* 7B2.

Circular Dichroism. The far-UV CD spectra of the 7B2 protein samples did not support the presence of typical features related to known secondary structures such as α helices or β strands (Figure 5). The characteristic negative peaks at 208 and 222 nm displayed by α helical proteins were not visible in 7B2 spectra. Estimation of secondary structure content revealed that all samples had a low α helical fraction (see Table 1). The α helical content was 29% for 27 kDa 7B2, while the β sheet content was 14%. The 21 kDa rat and the 27 kDa *C. elegans* proteins contained even fewer structural elements, with only 18% α helix and 26% β sheet content.

Disorder Prediction of 27 kDa 7B2. The CD and fluorescence findings were in accordance with predictions by PONDR-VL-XT (http://www.pondr.com/background.html), which identifies disordered regions in a protein. VL-XT predicts that 65% of the polypeptide chain of 27 kDa 7B2 and 50% of the *C. elegans* 7B2 amino acid sequence are

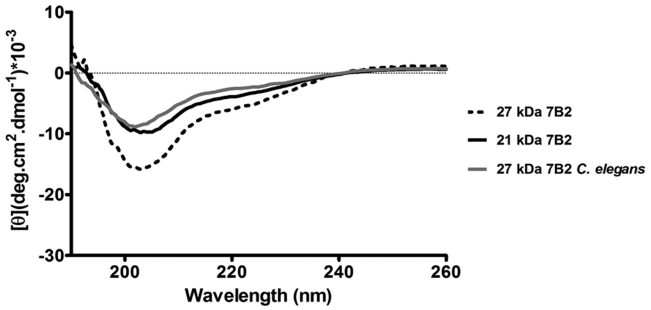

Figure 5. Far-UV CD spectra of 7B2 and variants. Wild-type rat 27 kDa 7B2 (black dashed line), wild-type rat 21 kDa 7B2 (black solid line), and 27 kDa *C. elegans* 7B2 (gray solid line) in 10 mM sodium phosphate and 0.4 mM octyl glucoside (pH 6.5).

Table 1. Secondary Structure Analysis of 7B2 Forms

7B2	% α helix	% β strand
27 kDa	29	14
21 kDa	18	26
27 kDa *C. elegans*	18	26

disordered. The longest disordered stretch in the rat 27 kDa 7B2 protein includes 40 residues between amino acid positions 73 and 112 (Figure 6). Such patterns of long unfolded regions lying between short structured segments are typical of intrinsically disordered proteins.[31]

NMR Spectroscopy. On the basis of the observations from the intrinsic tryptophan fluorescence and ANS binding experiments, we decided to evaluate the 27 kDa 7B2 protein using 2D NMR. The $^{1}H-^{15}N$ HSQC spectrum, shown in

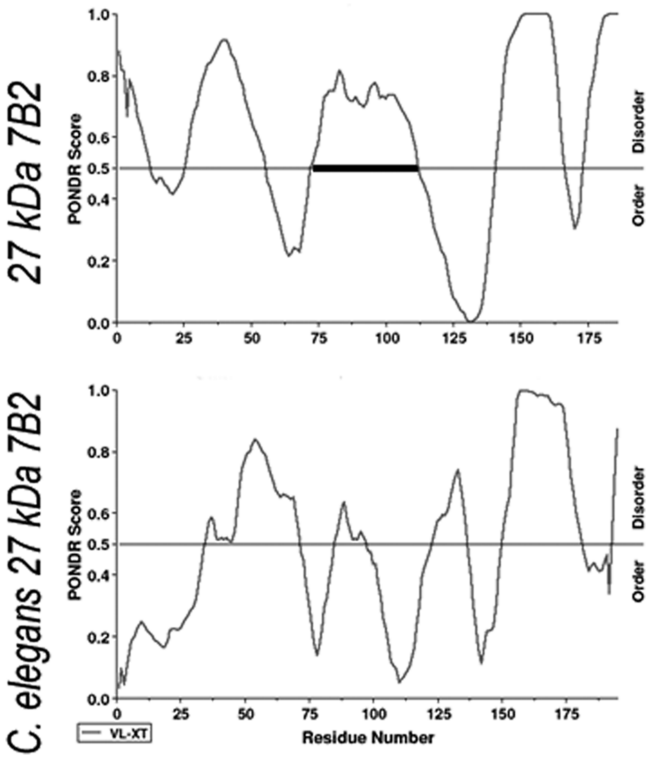

Figure 6. PONDR analysis of 27 kDa 7B2 and *C. elegans* 27 kDa 7B2 (signal peptides not included). The black bar shows a disordered region >40 residues in length.

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Figure 7, displayed a considerably smaller number of peaks than would be expected from a well-folded 186-residue protein. The

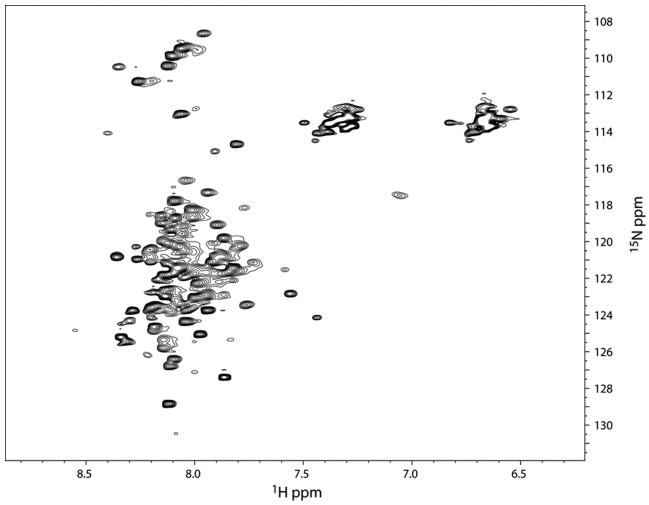

Figure 7. NMR of 27 kDa 7B2. These data depict the ^{1}H−^{15}N HSQC spectrum for 27 kDa 7B2.

chemical shift dispersion was also extremely narrow, between 6.9 and 8.4 ppm, which is indicative of unfolded protein. This conclusion was strengthened by the natural abundance ^{13}C−^{1}H HSQC spectrum, used to assess the environment around the core methyl groups, where we observed far fewer peaks and poor chemical shift dispersion (Figure 8) characteristic of

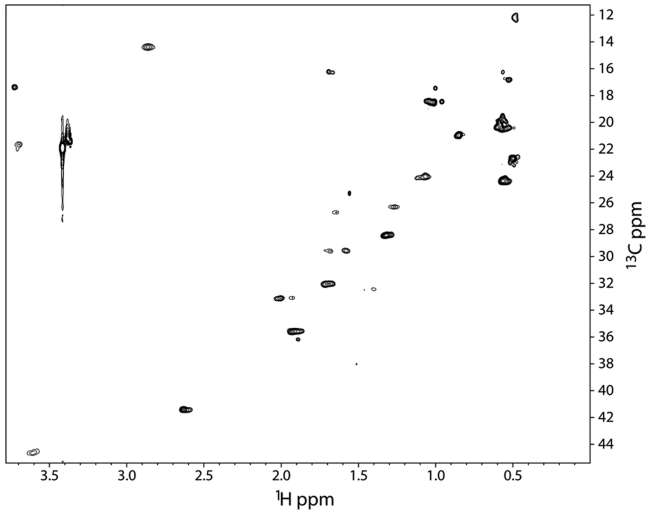

Figure 8. ^{13}C−^{1}H natural abundance carbon HSQC spectrum for 27 kDa 7B2.

unfolded protein. Together, these data support the CD results in showing that 7B2 lacks appreciable amounts of secondary structure. However, incubation of 27 kDa 7B2 with its putative client protein α-synuclein (Helwig et al., manuscript submitted for publication) resulted in the appearance of several new peaks as well as chemical shift perturbations for several of the peaks observed for 7B2 alone, indicating a change in protein conformation (marked with arrows in Figure 9). Complete assignment of the observed resonances is, however, necessary to confirm that new peaks are due to changes in 7B2 structure and not resonances within the central overlapped regions.

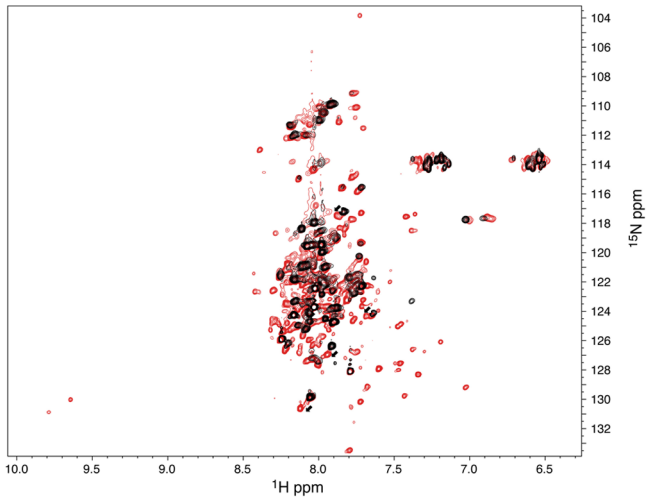

Figure 9. 7B2 in complex with client protein. ^{1}H−^{15}N TROSY spectrum for 27 kDa 7B2 complexed with α-synuclein. The black peaks were obtained from spectra acquired without α-synuclein, and the red peaks were obtained from spectra acquired after incubation with α-synuclein.

DISCUSSION

The small acidic 7B2 protein, expressed in all tissues containing a regulated secretory pathway (even those lacking proPC2 expression, for example, the thalamus[32]), was first described in 1982,[1,33] with N-terminal protein and cDNA sequence information provided between 1982 and 1991,[1,34−38] yet nothing is known of its three-dimensional structure to date. Recent data show that 7B2 can act as an antiaggregant chaperone for proPC2,[15] IGF-1,[14] and two neurodegeneration-related molecules, α-synuclein and Aβ 1−42. However, 7B2 is unable either to refold luciferase or to assist Hsp70 in refolding luciferase (Helwig et al., manuscript submitted for publication); thus, this protein does not function as a typical chaperone or cochaperone. In an effort to decipher the means by which 7B2 can interact with unrelated client proteins, we examined the secondary and tertiary structure of this protein.

A variety of biophysical techniques were used to demonstrate that both forms of 7B2 are natively disordered. Using intrinsic fluorescence, we found that although the 27 kDa form of 7B2 is somewhat susceptible to urea-induced unfolding, the 21 kDa form is relatively insensitive to changes in denaturant concentration, suggesting that the tryptophan residue in this form is already exposed to solvent in the native state. The relative folding propensities of the two forms were further characterized by ANS binding studies. A comparison of ANS binding to the 27 and 21 kDa forms of 7B2 under native conditions showed that ANS fluorescence increases by more than 40-fold upon binding the 27 kDa protein, but only 2-fold upon binding the 21 kDa protein. The 27 kDa protein thus has more accessible ANS-binding hydrophobic clusters than the 21 kDa form. It is known that ANS binds weakly to both the native and extensively unfolded states of proteins but binds quite strongly to solvent-accessible hydrophobic pockets in well-folded and partially folded stable intermediates such as the molten globule state.[39−41] On the basis of our ANS binding data, we infer that the 27 kDa 7B2 protein either contains a larger amount of tertiary structure than the 21 kDa form or has a higher degree of hydrophobic packing accessible to ANS, due to oligomerization.[42] CD spectroscopy also supported struc-

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tural differences between the 27 and 21 kDa forms; however, both proteins exhibited a very small amount of secondary structure. We conclude from these data that while the 27 kDa 7B2 protein is somewhat more compact than the 21 kDa form, overall, both proteins are mostly disordered.

Intrinsically disordered proteins are common in eukaryotes and are involved in the regulation of transcription, translation, and cell signaling.[43] A large number of disordered proteins are known to be associated with human disease (reviewed in ref 44); these proteins do not adopt stable tertiary structures under physiological conditions. Protein disorder is believed to provide a number of functional advantages, such as permitting multiple binding partners, a large surface area for partner binding, and providing considerable structural plasticity.[45] A special class of disordered proteins, known as elastomeric proteins, has been shown to be crucial for the elasticity of many biological tissues, e.g., the human arterial wall.[46] Understanding the physical and mechanistic basis of disorder-mediated functioning of unfolded proteins is currently a topic of active investigation.[44,47] In light of our previous findings that the 7B2 protein plays a critical role in blocking proPC2 aggregation and ensuing inability to activate,[15] as well as preventing fibrillation of several amyloidogenic proteins (Helwig et al., manuscript submitted for publication), we investigated a possible relationship between the structural properties of 7B2 and those of other molecular chaperones.

While no significant sequence homology exists between 7B2 and known chaperone proteins, the presence of structurally disordered regions is common among certain types of molecular chaperones, for example, α-crystallin and the small heat shock proteins (sHSP) Hsp 16.9, Hsp 25, and Hsp 33.[48,49] A known extracellular chaperone, clusterin, has been shown to colocalize with Aβ plaques in the brains of patients with Alzheimer's and with Lewy bodies in Parkinson's disease.[31] Similarly, we have demonstrated that 7B2 immunoreactivity colocalizes with Aβ plaques in an Alzheimer's model mouse (and in human Alzheimer's brain tissue), as well as with α-synuclein-rich Lewy bodies in Parkinson's brain tissue (Helwig et al., manuscript submitted for publication). Clusterin binds Aβ oligomers of all sizes, thereby influencing the rate of oligomer aggregation and disaggregation;[50] 7B2 blocks the fibrillation of both Aβ 1−40 and 1−42 (Helwig et al., manuscript submitted for publication). Lastly, clusterin is partially disordered and can bind several different proteins;[31] similarly, 7B2 also blocks the aggregation and fibrillation of a variety of unrelated proteins[14,15] (Helwig et al., manuscript submitted for publication). Our present data showing that 7B2 exists primarily as a disordered protein in a nonglobular, extended form support the idea that, like clusterin, 7B2 may possess a large surface area for binding multiple partners.

On the basis of the behavior of the 7B2 protein in gel filtration and cross-linking studies, we conclude that 7B2 is capable of forming dimers and multimers in a concentration-dependent manner. We confirmed the presence of multiple species (dimer, trimers, oligomers, etc.) in the 21 kDa 7B2 gel filtration peak, the amounts of which were proportional to the concentration of protein loaded onto the size exclusion column. This led us to hypothesize that a concentration-dependent dynamic equilibrium exists among the various 7B2 species. It was, however, surprising that the 7B2 protein, presumably consisting of a mixture of different oligomerization states, always eluted as a single peak during gel filtration chromatography. We postulate that a rapid, concentration-dependent equilibrium exists among the various species; coupled together with diffusion and dilution during gel filtration, this could result in the disappearance of the multiple peaks expected for the multimeric forms and the appearance of a single peak consisting of a mixture of all forms. Certain intrinsically disordered proteins have been shown to form dynamic homodimers that exist in rapid equilibrium between monomeric and dimeric forms.[51−53] 7B2's probable highly extended conformation, resulting in an increased hydrodynamic volume, may also contribute to its higher-than-expected apparent molecular mass during gel filtration. Interestingly, small heat shock proteins (sHSPs) are also known to form large oligomeric complexes that function to prevent protein aggregation.[54] We speculate that the antiaggregant effects of 7B2 can be attributed at least in part to this tendency toward multimerization, which has been regarded as a prerequisite for sHSP chaperone activity.[54,55] Further work is required to determine the size(s) of the 7B2 species that best bind client proteins.

Intrinsically disordered chaperones are known to exhibit distinct changes in secondary structure upon binding client proteins.[44] Consistent with this idea, we observed a shift in the conformational properties of the 27 kDa 7B2 protein when in complex with α-synuclein, suggesting induction of some structural features. Although α-synuclein is primarily a cytoplasmic protein, recent studies have reported detection of the protein in cerebrospinal fluid (CSF) and plasma.[56] The extracellular form of α-synuclein is believed to be delivered by exocytosis of α-synuclein and is the probable source of extracellular aggregates.[57] This mechanism may underlie the extracellular interaction between α-synuclein and secreted 7B2. In addition, unpublished data from our group (Helwig et al., manuscript submitted for publication) have shown that recombinant 7B2 can be taken up by Neuro2A cells, most likely by endocytosis. Thus, another possible mechanism of interaction between 7B2 and α-synuclein is re-uptake of 7B2 into an intracellular synuclein-containing compartment. Again, the mechanism of α-synuclein binding is unclear. Aggregation-prone proteins contain exposed hydrophobic surfaces that tend to associate.[48] The highly hydrophilic nature of disordered stretches within chaperone proteins provides a solubilization effect upon substrate binding that prevents these hydrophobic surfaces from approaching each other.[44,58,59] The disordered segments of the 7B2 protein may also function in this manner to block α-synuclein aggregation.

In conclusion, we show here that the small secretory protein 7B2 is an intrinsically disordered protein capable of oligomerization, thus exhibiting biophysical properties consistent with its proposed role as a secretory chaperone for misfolded neurodegeneration-related proteins.

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Notes

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■ ABBREVIATIONS

HSQC, heteronuclear single-nuclear quantum coherence spectroscopy; TROSY, transverse relaxation optimized spectroscopy; MALDI-TOF MS, matrix-assisted laser desorption time-of-flight mass spectrometry.

■ REFERENCES

(1) Hsi, K. L., Seidah, N. G., De Serres, G., and Chretien, M. (1982) Isolation and NH2-terminal sequence of a novel porcine anterior pituitary polypeptide. Homology to proinsulin, secretin and Rous sarcoma virus transforming protein TVFV60. *FEBS Lett. 147*, 261−266.

(2) Braks, J. A. M., and Martens, G. J. M. (1994) 7B2 is a neuroendocrine chaperone that transiently interacts with prohormone convertase PC2 in the secretory pathway. *Cell 78*, 263−273.

(3) Zhu, X., and Lindberg, I. (1995) 7B2 facilitates the maturation of proPC2 in neuroendocrine cells and is required for the expression of enzymatic activity. *J. Cell Biol. 129*, 1641−1650.

(4) Benjannet, S., Savaria, D., Chretien, M., and Seidah, N. G. (1995) 7B2 is a specific intracellular binding protein of the prohormone convertase PC2. *J. Neurochem. 64*, 2303−2311.

(5) Van Horssen, A. M., Van den Hurk, W. H., Bailyes, E. M., Hutton, J. C., Martens, G. J. M., and Lindberg, I. (1995) Identification of the region within the neuroendocrine polypeptide 7B2 responsible for the inhibition of prohormone convertase PC2. *J. Biol. Chem. 270*, 14292−14296.

(6) Zhu, X., Lamango, N. S., and Lindberg, I. (1996) Involvement of a polyproline helix-like structure in the interaction of 7B2 with prohormone convertase 2. *J. Biol. Chem. 271*, 23582−23587.

(7) Muller, L., Zhu, X., and Lindberg, I. (1997) Mechanism of the facilitation of PC2 maturation by 7B2: Involvement in ProPC2 transport and activation but not folding. *J. Cell Biol. 139*, 625−638.

(8) Zhu, X., Muller, L., Mains, R. E., and Lindberg, I. (1998) Structural elements of PC2 required for interaction with its helper protein 7B2. *J. Biol. Chem. 273*, 1158−1164.

(9) Muller, L., Zhu, P., Juliano, M. A., Juliano, L., and Lindberg, I. (1999) A 36-residue peptide contains all of the information required for 7B2-mediated activation of prohormone convertase 2. *J. Biol. Chem. 274*, 21471−21477.

(10) Ayoubi, T. A., van Duijnhoven, H. L., van de Ven, W. J., Jenks, B. G., Roubos, E. W., and Martens, G. J. (1990) The neuroendocrine polypeptide 7B2 is a precursor protein. *J. Biol. Chem. 265*, 15644−15647.

(11) Paquet, L., Bergeron, F., Boudreault, A., Seidah, N. G., Chretien, M., Mbikay, M., and Lazure, C. (1994) The neuroendocrine precursor 7B2 is a sulfated protein proteolytically processed by a ubiquitous furin-like convertase. *J. Biol. Chem. 269*, 19279−19285.

(12) Fortenberry, Y., Liu, J., and Lindberg, I. (1999) The role of the 7B2 CT peptide in the inhibition of prohormone convertase 2 in endocrine cell lines. *J. Neurochem. 73*, 994−1003.

(13) Mbikay, M., Seidah, N. G., and Chretien, M. (2001) Neuroendocrine secretory protein 7B2: Structure, expression and functions. *Biochem. J. 357*, 329−342.

(14) Chaudhuri, B., Stephan, C., Huijbregts, R. P., and Martens, G. J. (1995) The neuroendocrine protein 7B2 acts as a molecular chaperone in the in vitro folding of human insulin-like growth factor-1 secreted from yeast. *Biochem. Biophys. Res. Commun. 211*, 417−425.

(15) Lee, S. N., and Lindberg, I. (2008) 7B2 prevents unfolding and aggregation of prohormone convertase 2. *Endocrinology 149*, 4116−4127.

(16) Pasinetti, G. M., Ungar, L. H., Lange, D. J., Yemul, S., Deng, H., Yuan, X., Brown, R. H., Cudkowicz, M. E., Newhall, K., Peskind, E., Marcus, S., and Ho, L. (2006) Identification of potential CSF biomarkers in ALS. *Neurology 66*, 1218−1222.

(17) Finehout, E. J., Franck, Z., Choe, L. H., Relkin, N., and Lee, K. H. (2007) Cerebrospinal fluid proteomic biomarkers for Alzheimer's disease. *Ann. Neurol. 61*, 120−129.

(18) Bayes, A., and Grant, S. G. (2009) Neuroproteomics: Understanding the molecular organization and complexity of the brain. *Nat. Rev. Neurosci. 10*, 635−646.

(19) Jahn, H., Wittke, S., Zurbig, P., Raedler, T. J., Arlt, S., Kellmann, M., Mullen, W., Eichenlaub, M., Mischak, H., and Wiedemann, K. (2011) Peptide fingerprinting of Alzheimer's disease in cerebrospinal fluid: Identification and prospective evaluation of new synaptic biomarkers. *PLoS One 6*, e26540.

(20) Marcinkiewicz, M., Benjannet, S., Cantin, M., Seidah, N. G., and Chretien, M. (1986) CNS distribution of a novel pituitary protein '7B2': Localization in secretory and synaptic vesicles. *Brain Res. 380*, 349−356.

(21) Lindberg, I., Tu, B., Muller, L., and Dickerson, I. M. (1998) Cloning and functional analysis of *C. elegans* 7B2. *DNA Cell Biol. 17*, 727−734.

(22) Romero, P., Obradovic, Z., Li, X., Garner, E. C., Brown, C. J., and Dunker, A. K. (2001) Sequence complexity of disordered protein. *Proteins 42*, 38−48.

(23) Hwang, J. R., and Lindberg, I. (2001) Inactivation of the 7B2 inhibitory CT peptide depends on a functional furin cleavage site. *J. Neurochem. 79*, 437−444.

(24) Morrisett, J. D., David, J. S., Pownall, H. J., and Gotto, A. M., Jr. (1973) Interaction of an apolipoprotein (apoLP-alanine) with phosphatidylcholine. *Biochemistry 12*, 1290−1299.

(25) Chen, Y. H., Yang, J. T., and Chau, K. H. (1974) Determination of the helix and β form of proteins in aqueous solution by circular dichroism. *Biochemistry 13*, 3350−3359.

(26) Perez-Iratxeta, C., and Andrade-Navarro, M. A. (2008) K2D2: Estimation of protein secondary structure from circular dichroism spectra. *BMC Struct. Biol. 8*, 25.

(27) Iguchi, H., Chan, J. S., Seidah, N. G., and Chretien, M. (1987) Evidence for a novel pituitary protein (7B2) in human brain, cerebrospinal fluid and plasma: Brain concentrations in controls and patients with Alzheimer's disease. *Peptides 8*, 593−598.

(28) Iakoucheva, L. M., Kimzey, A. L., Masselon, C. D., Smith, R. D., Dunker, A. K., and Ackerman, E. J. (2001) Aberrant mobility phenomena of the DNA repair protein XPA. *Protein Sci. 10*, 1353−1362.

(29) Lackowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.

(30) Smoot, A. L., Panda, M., Brazil, B. T., Buckle, A. M., Fersht, A. R., and Horowitz, P. M. (2001) The binding of bis-ANS to the isolated GroEL apical domain fragment induces the formation of a folding intermediate with increased hydrophobic surface not observed in tetradecameric GroEL. *Biochemistry 40*, 4484−4492.

(31) Siltberg-Liberles, J. (2011) Evolution of structurally disordered proteins promotes neostructuralization. *Mol. Biol. Evol. 28*, 59−62.

(32) Seidel, B., Dong, W., Savaria, D., Zheng, M., Pintar, J. E., and Day, R. (1998) Neuroendocrine protein 7B2 is essential for proteolytic conversion and activation of proprotein convertase 2 in vivo. *DNA Cell Biology 17* (12), 1017−1029.

(33) Iguchi, H., Chan, J. S., Seidah, N. G., and Chretien, M. (1984) Tissue distribution and molecular forms of a novel pituitary protein in the rat. *Neuroendocrinology 39*, 453−458.
(34) Seidah, N. G., Hsi, K. L., De Serres, G., Rochemont, J., Hamelin, J., Antakly, T., Cantin, M., and Chretien, M. (1983) Isolation and NH2-terminal sequence of a highly conserved human and porcine pituitary protein belonging to a new superfamily. Immunocytochemical localization in pars distalis and pars nervosa of the pituitary and in the supraoptic nucleus of the hypothalamus. *Arch. Biochem. Biophys. 225*, 525−534.
(35) Brayton, K. A., Aimi, J., Qiu, H., Yazdanparast, R., Ghatei, M. A., Polak, J. M., Bloom, S. R., and Dixon, J. E. (1988) Cloning, characterization, and sequence of a porcine cDNA encoding a secreted neuronal and endocrine protein. *DNA 7*, 713−719.
(36) Martens, G. J. (1988) Cloning and sequence analysis of human pituitary cDNA encoding the novel polypeptide 7B2. *FEBS Lett. 234*, 160−164.
(37) Mbikay, M., Grant, S. G., Sirois, F., Tadros, H., Skowronski, J., Lazure, C., Seidah, N. G., Hanahan, D., and Chretien, M. (1989) cDNA sequence of neuroendocrine protein 7B2 expressed in β cell tumors of transgenic mice. *Int. J. Pept. Protein Res. 33*, 39−45.
(38) Waldbieser, G. C., Aimi, J., and Dixon, J. E. (1991) Cloning and characterization of the rat complementary deoxyribonucleic acid and gene encoding the neuroendocrine peptide 7B2. *Endocrinology 128*, 3228−3236.
(39) Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F., and Gilmanshin, R. I. (1991) Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers 31*, 119−128.
(40) Kumar, T. K., Subbiah, V., Ramakrishna, T., and Pandit, M. W. (1994) Trichloroacetic acid-induced unfolding of bovine pancreatic ribonuclease. Existence of molten globule-like state. *J. Biol. Chem. 269*, 12620−12625.
(41) Xu, Z., Xie, Q., and Zhou, H. M. (2003) Trichloroacetic acid-induced molten globule state of aminoacylase from pig kidney. *J. Protein Chem. 22*, 669−675.
(42) Frare, E., Mossuto, M. F., de Laureto, P. P., Tolin, S., Menzer, L., Dumoulin, M., Dobson, C. M., and Fontana, A. (2009) Characterization of oligomeric species on the aggregation pathway of human lysozyme. *J. Mol. Biol. 387*, 17−27.
(43) Dyson, H. J., and Wright, P. E. (2005) Intrinsically unstructured proteins and their functions. *Nat. Rev. Mol. Cell Biol. 6*, 197−208.
(44) Tompa, P. (2002) Intrinsically unstructured proteins. *Trends Biochem. Sci. 27*, 527−533.
(45) Chen, J. (2012) Towards the physical basis of how intrinsic disorder mediates protein function. *Arch. Biochem. Biophys. 524*, 123−131.
(46) Rauscher, S., and Pomes, R. (2012) Structural disorder and protein elasticity. *Adv. Exp. Med. Biol. 725*, 159−183.
(47) Fink, A. L. (2005) Natively unfolded proteins. *Curr. Opin. Struct. Biol. 15*, 35−41.
(48) Tompa, P., and Csermely, P. (2004) The role of structural disorder in the function of RNA and protein chaperones. *FASEB J. 18*, 1169−1175.
(49) Reichmann, D., Xu, Y., Cremers, C. M., Ilbert, M., Mittelman, R., Fitzgerald, M. C., and Jakob, U. (2012) Order out of disorder: Working cycle of an intrinsically unfolded chaperone. *Cell 148*, 947−957.
(50) Narayan, P., Orte, A., Clarke, R. W., Bolognesi, B., Hook, S., Ganzinger, K. A., Meehan, S., Wilson, M. R., Dobson, C. M., and Klenerman, D. (2012) The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid-β(1−40) peptide. *Nat. Struct. Mol. Biol. 19*, 79−83.
(51) Danielsson, J., Liljedahl, L., Barany-Wallje, E., Sonderby, P., Kristensen, L. H., Martinez-Yamout, M. A., Dyson, H. J., Wright, P. E., Poulsen, F. M., Maler, L., Graslund, A., and Kragelund, B. B. (2008) The intrinsically disordered RNR inhibitor Sml1 is a dynamic dimer. *Biochemistry 47*, 13428−13437.
(52) Lanza, D. C., Silva, J. C., Assmann, E. M., Quaresma, A. J., Bressan, G. C., Torriani, I. L., and Kobarg, J. (2009) Human FEZ1 has characteristics of a natively unfolded protein and dimerizes in solution. *Proteins 74*, 104−121.
(53) Sigalov, A. B. (2012) Interplay between protein order, disorder and oligomericity in receptor signaling. *Adv. Exp. Med. Biol. 725*, 50−73.
(54) Leroux, M. R., Melki, R., Gordon, B., Batelier, G., and Candido, E. P. (1997) Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. *J. Biol. Chem. 272*, 24646−24656.
(55) Giese, K. C., and Vierling, E. (2002) Changes in oligomerization are essential for the chaperone activity of a small heat shock protein in vivo and in vitro. *J. Biol. Chem. 277*, 46310−46318.
(56) Kim, K. S., Choi, Y. R., Park, J. Y., Lee, J. H., Kim, D. K., Lee, S. J., Paik, S. R., Jou, I., and Park, S. M. (2012) Proteolytic Cleavage of Extracellular α-Synuclein by Plasmin: Implications for Parkinson Disease. *J. Biol. Chem. 287*, 24862−24872.
(57) Lee, H. J., Patel, S., and Lee, S. J. (2005) Intravesicular localization and exocytosis of α-synuclein and its aggregates. *J. Neurosci. 25*, 6016−6024.
(58) Lindner, R. A., Carver, J. A., Ehrnsperger, M., Buchner, J., Esposito, G., Behlke, J., Lutsch, G., Kotlyarov, A., and Gaestel, M. (2000) Mouse Hsp25, a small shock protein. The role of its C-terminal extension in oligomerization and chaperone action. *Eur. J. Biochem. 267*, 1923−1932.
(59) Uversky, V. N. (2002) Natively unfolded proteins: A point where biology waits for physics. *Protein Sci. 11*, 739−756.