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Probing the Secondary Structure of a Recombinant Neuronal Adaptor Protein and Its Phosphotyrosine Binding Domains

Annalisa LAMBERTI,[1] Olimpia LONGO,[1] Pompea Del VECCHIO,[3] Nicola ZAMBRANO,[1,2] Guido BARONE,[3] Tommaso RUSSO,[1,2] and Paolo ARCARI[1,2,†]

[1]*Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Via S. Pansini 5, I-80131 Napoli, Italy*

[2]*CEINGE, Biotecnologie Avanzate scarl, Via Comunale Margherita, I-80131 Napoli, Italy*

[3]*Dipartimento di Chimica, Università di Napoli Federico II, Via Cintia 45, Monte S. Angelo, I-80126 Napoli, Italy*

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Rat brain Fe65 and its truncated forms corresponding to the combined PTB1 and PTB2 domains, as well as to the isolated PTB2 domain, were expressed in *Escherichia coli* and purified from inclusion bodies by affinity chromatography. The recombinant proteins were refolded and judged functionally active by their ability to interact with native APP. Limited proteolysis of recombinant Fe65 and PTB1–2 with trypsin, chymotrypsin and V8 proteases showed that the most sensitive proteoltytic sites were positioned at the level of the interdomain regions comprised between WW/PTB1 and PTB1/PTB2. Secondary structure of the recombinant proteins, evaluated by CD spectroscopy, showed a different degree of unordered structures, the PTB2 domain being the higher organised region. In addition, intrinsic fluorescence measurements of PTB2, indicated that a conformational transition of the protein can be induced by denaturating agents such as GuHCl. These data provide first evidences on the secondary structural levels of Fe65.

Key words: rat brain Fe65; PID/PTB domain; recombinant proteins; limited proteolysis; circular dichroism

Fe65 is an adaptor protein expressed in neurons of several regions of the mammalian nervous system.[1,2] Its gene belongs to a mammalian multigene family composed of three different members; its relevance during evolution is justified by the presence of a single orthologue gene in the nematode *Caenorhabditis elegans*.[3] The encoded proteins contain two different types of protein-protein interaction domains: a WW and two phosphotyrosine binding (PTB)-related domains. Rat Fe65 is made of 711 amino acid residues; its WW domain (aa 254–288) interacts with several proteins, like Mena, the mammalian orthologue of Drosophila enabled protein, and the non-receptorial tyrosine kinase Abl.[4,5] The two phosphotyrosine binding domains PTB1 (aa 365–510) and PTB2 (aa 538–665) are responsible for the interaction of Fe65 with membrane and nuclear proteins: the PTB1 domain binds the CP2/LSF/LBP1 transcription factor[6] and the histone acetyl transferase Tip60[7] while the PTB2 domain is responsible for the interaction of Fe65 with the cytosolic, carboxy terminal region of the Alzheimer's β-amyloid precursor protein (APP), through the consensus motif NPXY.[8] The N-terminal region of the protein (aa 1–253) does not contain obvious protein domains, shows no relevant identities to any known protein, and is characterised by a high content of acidic amino acids, also found in a class of nuclear proteins containing a HMG-like (High Mobility Group I) acidic region.[9] The interaction of Fe65 with APP[10] has important functional consequences because it is implicated in the pathogenetic mechanisms of Alzheimer's disease. In fact, APP is a membrane protein that upon proteolytic processing produces the Alzheimer β-amyloid peptide (Aβ)[11] the main constituent of the senile plaques of Alzheimer disease.[12,13] This process is affected by the interaction of APP with other two proteins containing a PTB domain: X11 and mDab1.[14,15] Overexpression of Fe65 determines in cell cultures an increment of Aβ[16,17] whereas the overexpression of X11 inhibits the APP processing.[18–20] Despite the knowledge on the functional aspects of Fe65, not so much is known about its structural properties. In this work, we analysed recombinant rat Fe65 protein and its truncated forms containing both phosphotyrosine binding domains (PTB1–2), or the single PTB2 domain by means of biochemical and

† To whom correspondence should be addressed. Fax: +39-081-7463653; E-mail: arcari@dbbm.unina.it

Abbreviations: aa, amino acids; APP, amyloid precursor protein; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; CD, circular dichroism; FPLC, fast protein liquid chromatography; GST, glutathione S-transferase; GuHCl, guanidine hydrochloride; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni–NTA, Nickel–NitroaceticAcid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PTB domain, phosphotyrosine binding domain; WW domain, domain binding protein

spectroscopic methods. The results obtained provide first structural data on the secondary structure of Fe65.

Materials and Methods

Plasmid construction, expression and purification of intact and truncated Fe65. Plasmid containing full length Fe65 rat cDNA (Clone 21)[1] was used as template in PCRs in order to prepare DNA fragments corresponding to intact Fe65 and to the regions containing PTB1–2 and PTB2 domains. The following forward primers were used, respectively: (Fe1) 5′-CGCAGGATCTACTAAGCATATGTC, (Fe8) 5′-GAGAATCTGCCCCATATGAACGC and (Fe7) 5′-ACTCTAAACATATGGATGTCCCTT all containing an NdeI restriction site; the reverse primer 5′-CTACCTGCCCCAAAAGCTTGAG (Fe5), which contained a HindIII site, was common to all three constructs. The PCR products were first inserted into pGEM-T Easy and subsequently cloned into the NdeI-HindIII sites of pET22b(+) expression vector (Novagen).[21,22] Recombinant plasmids were then used to transform *E. coli* BL21(DE3). Cells were grown up to 0.6 A.U. and induced with 100 mg/ml IPTG (Inalco) for 3 h. After centrifugation at 5000 rpm for 15 min, cells were resuspended in lysis buffer (20 mM Tris HCl, pH 7.8, 50 mM EDTA, 1 mM β-mercaptoethanol for Fe65; 20 mM Tris HCl, pH 6.8 for PTB1–2 and PTB2) and disrupted in a cell disruption system (Constant Systems, Ltd., UK) at 1.5 kbar. Samples were then centrifuged at 10,000 rpm ($28{,}000 \times g$) for 30 min, and the recombinant proteins were solubilized and purified from inclusion body according the following procedures: pellets from 1 liter of cell culture were first dissolved in 50 ml of 8 M urea, centrifuged at 10,000 rpm for 30 min and then extensively dialysed against 20 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM β-mercaptoethanol, 0.5 mM EDTA and 10% glycerol in the case of intact Fe65 whereas, in the case of PTB1–2 and PTB-2, were first rapidly diluted 1:1 with a buffer containing 20 mM Tris HCl, pH 9 and 0.8 M arginine and then dialysed against 10 mM Na_2HPO_4, pH 10, 1 mM arginine, 1.5 mM cysteine and 0.5 mM cystine.[23] Samples were loaded onto a Ni–NTA Superflow (Qiagen) column (2×20 cm), connected to a FPLC system (Pharmacia), equilibrated with 50 mM Na_2HPO_4 pH 8, 300 mM NaCl, 10 mM imidazole, in the case of intact Fe65, or 50 mM Na_2HPO_4 pH 10, 300 mM NaCl, 10 mM imidazole, in the case of PTB1–2 and PTB2. After extensive washes with the above buffers containing 50 mM imidazole (10 column volumes), proteins were eluted raising the imidazole concentration up to 200 mM. Fraction containing a single protein band on SDS–PAGE stained with Coomassie Brilliant Blue were pooled, concentrated with Aquacide IIa and dialysed against 10 mM Tris HCl pH 8, in the case of intact Fe65, and 10 mM Na_2HPO_4 pH 10, in the case of PID1–2 and PID2 and stored at $-20\,^{\circ}$C. Protein concentration was determined by the method of Lowry.[24]

Antibody, pull down and western blots. Fe65 or PTB1–2 and PTB2 constructs were detected with anti-His-Tag monoclonal antibodies (Santa Cruz), anti-Fe65 serum (I12) and anti-PTB1–2 serum (E9), raised in rabbit.[25] Pull down were performed according the following protocol: GST-Sepharose resin equilibrated in PBS 1×, 1 mM DTT (35 ml), was incubated with 10 µg of GST–APP,[25] or with GST as control, and after two 5 min washes with the equilibration buffer, each sample was incubated at 4 °C for 2 hours with 200 ng of purified PTB2, or with lysates. Samples, after three 5 min washes at 4 °C with 20 mM Na_2HPO_4 pH 10 were denatured and resolved on SDS–PAGE for western blotting. Proteins were then transferred to Immobilion P membranes (Millipore), blocked in 5% non-fat dry milk in PBS-T for 1–2 hours at 4 °C and incubated with appropriate dilution of the primary antibody for 2 h at room temperature. The excess antibody was removed by sequential washing of the membranes in PBS-T, and then a 1:20,000 dilution of horsereadish peroxidase-conjugated protein A (Amersham Pharmacia Biotech) was added to the filters for 1 h at room temperature. Filters were washed and the signals detected by chemiluminescence using the Super Signal West Pico (Pierce).

Proteolysis. Samples of purified intact Fe65, or PTB1–2 and PTB2 (80 µg), were incubated in 20 mM Tris–HCl pH 7.8, 1 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercaptoetanol with 0.8 µg of trypsin, chymotrypsin or V8 in a final volume of 80 µl. At the indicated times, 20 µl aliquots were withdrawn from the reaction mixture and analysed on SDS–PAGE[26] or by Western blot. N-terminal amino acid sequence of the products was determined after transfer on membrane for microsequences (Problot) in CAPS by an automatic sequencer (Mod 473 A, Applied Biosystem) connected on line to a HPLC apparatus for PTH-amino acid identification.

CD and fluorescence spectroscopy. CD spectra, recorded in a Jasco J-715 spectropolarimeter equipped with a Peltier type temperature control system (Model PTC-348WI), at time constant of 16 s, 2 nm band width, and scan rate of 5 nm min^{-1}, were signal-averaged over at least five scans, and baseline corrected by subtracting a buffer spectrum. The instrument was calibrated with an aqueous solution of d-10-(+)-camphorsulfonic acid at 290 nm.[27] Molar ellipticity per mean residue, $[\theta]$ in $deg \cdot cm^2 \cdot dmol^{-1}$, was calculated from the equation: $[\theta] = [\theta]_{obs}\, mrw/10 \cdot l \cdot C$, where $[\theta]_{obs}$ is the ellipticity measured in millidegrees, mrw is the mean residue molecular weight, C is the protein concentration in $g \cdot l^{-1}$ and l is the optical path length of the cell in cm. A 0.1 cm path length cell and a protein concentration ranged from 0.05 to 0.1 $mg \cdot ml^{-1}$ were used. Fluorescence spectra were recorded in a Perkin Elmer LS50B spectrofluorimeter equipped with a Perkin Elmer PTP-1 Peltier System, at 10 °C, 1 cm sealed cell, 5 nm emission

slit width and corrected for background signal. The excitation wavelength was set at 290 nm and the protein concentration ranged from 0.02 to 0.04 mg·ml^{-1}. The protein concentration for CD and fluorescence measurements was determined spectrophotometrically using theoretical, sequence-based[28] extinction coefficients of 98,400 M^{-1}·cm^{-1}, 59,000 M^{-1}·cm^{-1} and 27,500 M^{-1}·cm^{-1} at 280 nm for Fe65, PTB1–2 and PTB2, respectively.

Results and Discussion

Purification of intact Fe65, PTB1–2 and PTB2 domains

Intact recombinant rat brain Fe65 (M_r 79,048) and its isolated PTB1–2 (aa R362-P711, M_r 40,045) and PTB2 (aa V525-P711, M_r 21,799) were purified from *E. coli* inclusion bodies by affinity chromatography on Ni–NTA Sepharose (Fig. 1). Recombinant proteins were identified, in the case of Fe65, by sequencing the N-terminal region and by Western blot with antibodies anti-WW and anti-His-Tag whereas, in the case of PTB1–2 and PTB2, by interaction with antibodies anti-PTB2 and anti-His-Tag (not shown). Because Fe65 and its PTB domains do not have enzyme activity, to assess if the refolding procedures were efficient, we tested their ability to interact with the cytosolic region of APP by pull-down. As reported in Fig. 2, both antibodies anti-WW and anti-PTB2 were able to precipitate the GST–APP•Fe65 and GST–APP•PTB2 complexes, respectively, thus suggesting an appropriate refolding of the recombinant proteins (Fig. 2). Same result was obtained with recombinant PTB1–2 (not shown). All three proteins resulted soluble if the concentration was not higher than 0.1–0.2 mg/ml. Above this value, aggregates were observed.

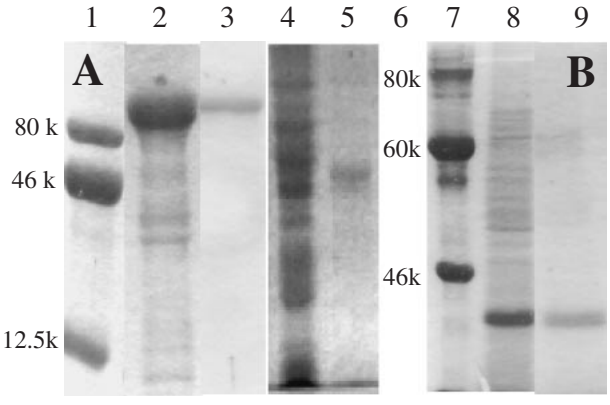

Fig. 1. SDS–PAGE of His-Tagged Fe65, PTB1–2 and PTB2 Purification Steps.

A, 10% acrylamide gel loaded with: 1) molecular weight markers, 2) 25 µg P30 extract (Fe65), 3) 2.5 µg of purified Fe65, 4) 50 µg P30 extract (PTB1–2), 5) 2 µg of purified PTB1–2. B, 12% acrylamide gel loaded with: 6) molecular weight markers, 7) 25 µg P30 extract (PTB2), 8) 2.5 µg of purified PTB2.

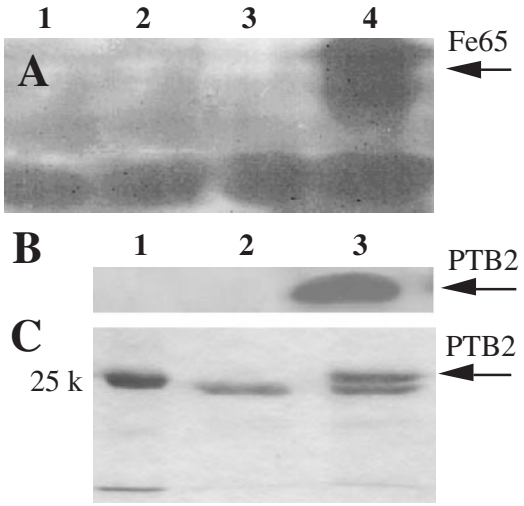

Fig. 2. Pull Down of Intact Fe65 and Its PTB2 Form.

A, 20 µl Glutathione-Sepharose slurry were saturated with: 1) 2.5 µg wild type GST, 2) 2.5 µg GST in presence of 0.1 µg of intact Fe65, 3) 2.5 µg GST–APP, 4) 2.5 µg GST–APP in presence of 0.1 µg of intact Fe65. Each Sample was incubated for 2 h at 4 °C and after 4 washes in 20 mM phopsphate buffer, pH 10, was centrifuged and the resin was loaded onto a 10% polyacrilamide gel. Western blot assay was done with anti-WW (I12). B and C, 20 µl Glutathione-Sepharose slurry were saturated with: 1) 5 µg GST in presence of 0.2 µg of PTB2, 2) 5 µg GST–APP in presence of 0.2 µg of PTB2. Samples were incubated at 4 °C for 2 h and after 4 washes in 20 mM phopsphate buffer, pH 10, they were centrifuged, loaded on 10% polyacrilamide gel and assayed by (B) Western blot with anti-PTB1–2 (E9), (C) coomassie blue staining.

Limited proteolysis

Figure 3 shows the time course of the proteolytic cleavege of Fe65 with chymotrypsin, V8 and trypsin. Chymotrypsin produced two major fragments of about 60 and 19 kDa. The formation of the proteolytic products was accompanied by the disappearance of intact Fe65 (Fig. 3A). Edman degradation of the larger fragment gave the N-terminal sequence of the intact protein, whereas the sequence of 19 kDa fragment showed the presence of a proteolytic site at the level of Phe544. Limited proteolysis of intact Fe65 with V8 yielded the formation of two major peptides of about 50 and 35 kDa (Fig. 3B). The sequence of the 35 kDa fragment contained the N-terminal sequence whereas the larger fragment indicated an exposed site in correspondence of Glu298. Proteolysis with trypsin (Fig. 3C) showed the formation of two major fragments of about 60 and 25 kDa. Sequencing of the larger fragment indicated the presence of a proteolytic sites at the level of Arg214, whereas no sequence was possible to collect with the other fragment. Also the recombinant PTB1–2 was analysed by digestion with the same proteases. In the case of trypsin and V8, no discrete products were detectable on SDS–PAGE. Using instead chymotrypsin with a ratio protease/PTB1–2 of 1:150 w/w, two products were detected on SDS–PAGE of approximately 20 and 16 kDa (not shown). The size of the fragments and their recognition by the specific anitibodies anti-

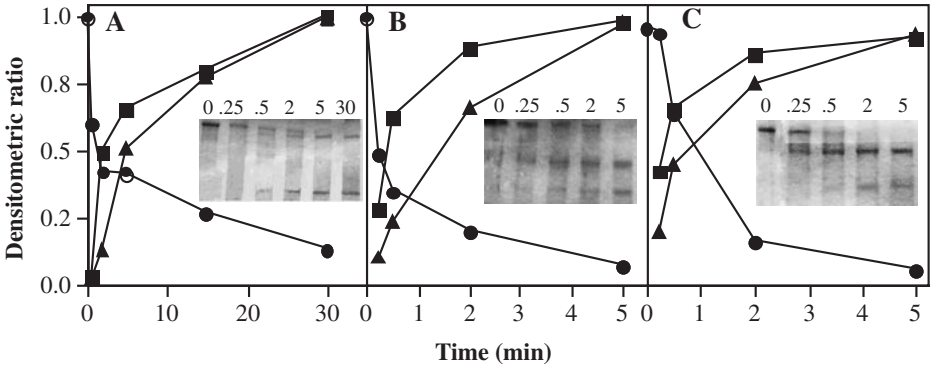

Fig. 3. SDS–PAGE of Fe65 Digested with Chymotrypsin, V8 and Trypsin.

Aliquots of Fe65 at different incubation times were withdrawn from the reaction mixture and analysed on SDS–PAGE (insets). A, Chymotrypsin digestion at 0 °C: (●) Intact Fe65, (■) 60 kDa band, (▲) 19 kDa band. B, V8 digestion at 37 °C: (●) Intact Fe65, (■) 50 kDa band, (▲) 35 kDa band. C, Trypsin digestion at 37 °C: (●) Intact Fe65, (■) 60 kDa and (▲) 25 kDa band. Gels were scanned and bands densitometry were normalized.

PTB2 and anti-His-Tag, respectively suggested the presence of a cleavage site between PTB1 and PTB2 (Phe544), as well as of another proteolytic site at the level of one of the aromatic amino acid present in the C-terminal sequence after PTB2 domain (most likely Trp698), as deduced by the size of the small fragment detected by anti-His-Tag (Fig. 4). These results are in agreement with those obtained by digestion of the intact Fe65 with chymotrypsin, thus confirming the presence of an exposed site between PTB1 and PTB2 domains. Figure 5 shows the positions of the exposed sites localised between the regions of Fe65 containing the WW domain and the phosphotyrosine interacting domains PTB1 and PTB2.

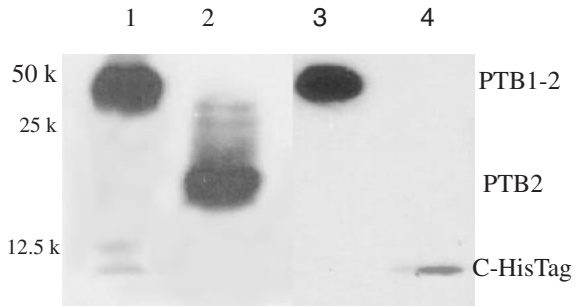

Fig. 4. Western Blot of PTB1–2 Digested with Chymotrypsin.

PTB1–2 was incubated with 0.5 µg of chymotrypsin at 0 °C for 5 min. The reaction was stopped with 2× loading buffer and 20 µl aliquots were loaded on 12% SDS–PAGE and analysed by Western blot using anti-PTB2 (E9) (lane 1, zero min; lane 2, 5 min incubation) or using anti-His-Tag (lane 3, zero min; lane 4, 5 min incubation).

Circular dichroism and fluorescence measurements

Secondary structure of recombinant Fe65 and of PTB1–2 and PTB2 domains was investigated by means of CD in the far-UV region. Figure 6 shows the CD spectra of the intact Fe65, PTB1–2 and PTB2 domains recorded in a phosphate buffer 10 mM pH 10. The spectra suggested that PTB1–2 and PTB2 domains displayed a higher content of secondary structure compared to the intact protein. In fact, the more intense negative band near 200 nm present in Fe65 is indicative of unordered structures, whereas in PTB2, the CD spectrum showed a large negative band at 222 nm. The estimation of the secondary structure of Fe65, PTB1–2 and PTB2 performed on the basis of the CD spectra, according to the Variable Selection Method (CDSSTR),[29] by using DICHROWEB[30] is reported in Table 1. The level of secondary structure found in PTB2 was compared to those of the corresponding PTB domain of X11 (aa R324-G491) and Shc (aa H40-P200) as evaluated from their 3D structures, respectively.[31,32] Despite the very low amino acid sequence identities between these domains,[3] PTB2 displayed a comparable content of secondary structures. In fact, X11 and Shc showed 58% and 54% secondary structures, respectively, with a content of α-helix (26% and 23%, respectively). These results suggested that PTB2 domain possesses a folded structure in solution. In order to evidentiate the folded state of PTB2 domain, we studied

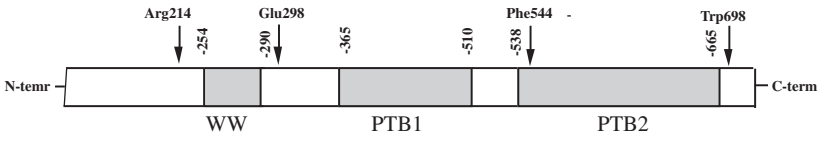

Fig. 5. Cleavage Sites in Fe65.

The position of the sites are reported in relation to the structural domains present in the protein.

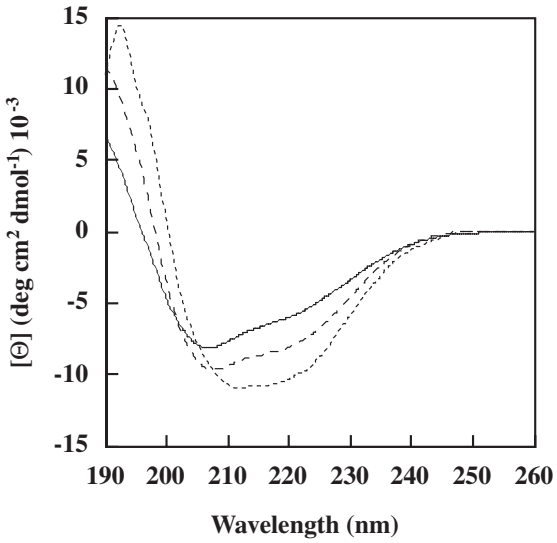

Fig. 6. Far-UV CD Spectra of Recombinant His-Tagged Fe65, PTB1–2 and PTB2.

Measurements were recorded at 25 °C in 10 mM phosphate buffer, pH 10. Continuous line, Fe65; dashed line, PTB1–2; dotted line, PTB2.

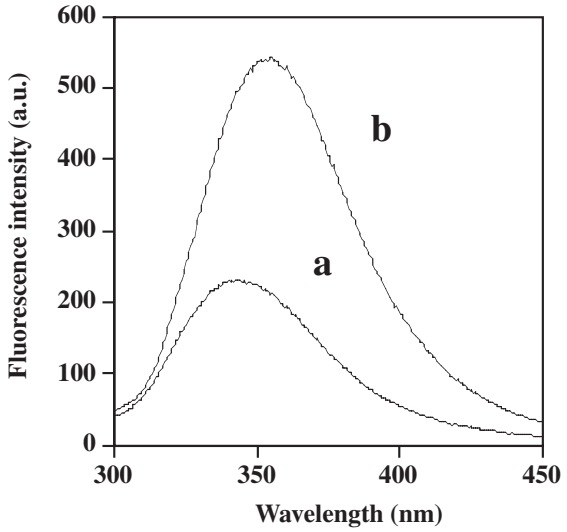

Fig. 7. Fluorescent Emission Spectra of Native and Unfolded PTB2. Spectra were recorded phosfate buffer 10 mM, pH 10 at 20 °C in the absence (a) or in the presence (b) of 6 M GuHCl.

Table 1. Estimation of Secondary Structure in Recombinant Fe65 and Its Truncated Forms PTB1–2 and PTB2

Protein	% Helix	% Strand	% Turns	% Unordered
Fe65	5	23	18	50
PTB1–2	10	28	20	45
PTB2	19	27	21	33

the GuHCl-induced denaturation. Figure 7 shows the comparison of the fluorescence of PTB2 in the absence and presence of 6 M GuHCl, respectively. The emission spectra were registered upon excitation at 290 nm in order to eliminate the fluorescence tyrosine contribution. In the absence of the denaturating agent, PTB2 domain showed the maximum of emission intensity at 342 nm that shifts to 352 nm, in the presence of 6 M GuHCl. A maximum around 335 nm is characteristic of tryptophan residues well buried in the hydrophobic core, whereas fluorescence spectra with a maximum around 350 nm are characteristic of tryptophan residues exposed to the aqueous solution.[33] The spectra indicated that PTB2 domain have the tryptophan residues buried in the native structure, and well exposed in the GuHCl-induced unfolded state. The results obtained by fluorescent spectra gave an indication that PTB2 domains have a defined conformational structure in solution.

In conclusion, in this work we studied the properties of recombinant rat brain Fe65 and of its isolated phosphotyrosine binding domains PTB1–2 and PTB2, with the aim of obtaining insights about its structure. The expression and purification system adopted was suitable to produce functional recombinant proteins. Although the maximum possible concentration of the refolded proteins was not high, it was sufficient to carry out some structural investigations using limited proteolysis and spectroscopic methods. The results obtained showed that: *i*) the most exposed proteolytic sites were found localised between the regions of Fe65 containing the WW domain and the phosphotyrosine interacting domains PTB1 and PTB2. These findings are in agreement with the general observation that limited proteolysis of native globular proteins generally occurs at the level of the inter-domain regions and in particular at hinges and fringes of the polypeptide chain;[34] *ii*) the data collected by CD spectroscopy suggested in particular that PTB2 domain represents a region of the protein with high secondary structure levels.

Acknowledgments

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