Published in final edited form as:

Nat Struct Mol Biol. 2013 April ; 20(4): 495–501. doi:10.1038/nsmb.2503.

Characterization of prion-like conformational changes of the neuronal isoform of *Aplysia* CPEB

Bindu L Raveendra^{1,5}, Ansgar B Siemer^{2,5}, Sathyanarayanan V Puthanveetil^{1,3,5}, Wayne A Hendrickson^{3,4}, Eric R Kandel^{1,3,4}, and Ann E McDermott²

¹Department of Neuroscience, Columbia University, New York, New York, USA

²Department of Chemistry, Columbia University, New York, New York, USA

³Howard Hughes Medical Institute, Columbia University, New York, New York, USA

⁴Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York, USA

Abstract

The neuronal isoform of cytoplasmic polyadenylation element-binding protein (CPEB) is a regulator of local protein synthesis at synapses and is critical in maintaining learning-related synaptic plasticity in *Aplysia*. Previous studies indicate that the function of *Aplysia* CPEB can be modulated by conversion to a stable prion-like state, thus contributing to the stabilization of long-term memory on a molecular level. Here, we used biophysical methods to demonstrate that *Aplysia* CPEB, like other prions, undergoes a conformational switch from soluble α -helix-rich oligomer to β -sheet-rich fiber *in vitro*. Solid-state NMR analyses of the fibers indicated a relatively rigid N-terminal prion domain. The fiber form of *Aplysia* CPEB showed enhanced binding to target mRNAs as compared to the soluble form. Consequently, we propose a model for the *Aplysia* CPEB fibers that may have relevance for functional prions in general.

Although significant knowledge of cellular and molecular mechanisms underlying the acquisition and early storage of implicit and explicit long-term memory has been gained, the mechanisms by which memories are maintained for long periods of time are still not fully understood^{1,2}. Because proteins normally have relatively short half-lives, of hours or days, the question remains: How can the change in molecular composition of a synapse be maintained for long periods of time, as is required for long-term memory? We previously

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Correspondence should be addressed to E.R.K. (erk5@columbia.edu) or A.E.M. (aem5@columbia.edu).

⁵Present addresses: Department of Chemistry, Scripps Research Institute, Jupiter, Florida, USA (B.L.R.), Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, California, USA (A.B.S.) and Department of Neuroscience, Scripps Research Institute, Jupiter, Florida, USA (S.V.P.)

Note: Supplementary information is available in the online version of the paper.

Author Contributions

B.L.R., S.V.P. and A.B.S. designed the project after discussing with E.R.K., A.E.M. and W.A.H. All the experiments were done by B.L.R. except NMR and RNA-binding experiments. NMR experiments were done by A.B.S. and RNA-binding experiments by S.V.P. B.L.R., A.B.S., E.R.K. and A.E.M. wrote the paper.

Competing Financial Interests

The authors declare no competing financial interests.

found one answer to this conundrum in a work describing a prion-like regulator of local protein synthesis at the synapse in the marine snail *Aplysia californica*: the cytoplasmic polyadenylation element-binding protein *Aplysia* CPEB^{3,4}. This provided physiological evidence that the prion-like properties of *Aplysia* CPEB might explain the self-sustained, continuous molecular turnover at the synapse⁵.

Generally, CPEB proteins bind a six-nucleotide sequence, the cytoplasmic polyadenylation element (CPE) at the 3' untranslated regions (3' UTRs) of mRNAs, and recruit the polyadenylation machinery required for translational activation^{6,7}. The neuronal isoform of *Aplysia* CPEB differs from other isoforms of CPEB in having an N-terminal domain that is rich in glutamine and asparagine⁸, similar to the QN-rich domain of certain yeast prions (Sup35p, Ure2p and Rnq1p)^{9–11}. When prions convert from soluble form to self-perpetuating infectious form, they undergo a conformational change from a globular or an unstructured form to an aggregated β -sheet-enriched amyloid structure^{12–14}. Previous studies have shown that *Aplysia* CPEB exists in at least two different physical conformations, one of which is an aggregated self-perpetuating form^{3,5} (Fig. 1). Moreover, *in vitro*-formed fibers of isolated prion domains of *Aplysia* CPEB are able to induce conformational changes and can self-perpetuate in yeast cells¹⁵. These findings are consistent with the idea that the N-terminal QN-rich extension confers a new ability upon *Aplysia* CPEB to function as a biologically useful prion in the nervous system. Recent evidence indicates that the aggregated form of *Aplysia* CPEB is in the active conformation and is essential for the maintenance of long-term synaptic plasticity in *Aplysia*⁵. The homologs of CPEB in *Drosophila* (Orb2)¹⁶ and in mice (mCPEB3)¹⁷ have similarly been found to be essential for long-term memory. The potential generality of this hypothesis is underscored by the identification of isoforms of CPEB with QN-rich terminal regions in humans¹⁸.

To date, to our knowledge, there has been no direct biophysical evidence for the existence of *Aplysia* CPEB in two forms similar to the different conformational states of other known prions. Here we demonstrate that *Aplysia* CPEB can exist in at least two different conformational forms: an α -helix-rich form and a β -sheet-rich amyloid form. Our solid-state NMR results indicate that the prion-like N-terminal domain of *Aplysia* CPEB is not entirely composed of β -sheets but also has helical and random-coil signatures. We propose a model for the aggregated form of *Aplysia* CPEB in which the N-terminal domains form the fiber axis, whereas the RNA-binding domains project out of the fiber axis.

RESULTS

The soluble form of *Aplysia* CPEB exists as oligomers

To characterize the different conformational forms of *Aplysia* CPEB *in vivo*, we used total soluble-protein extract from the central nervous system (CNS) of *Aplysia* under native conditions. The *Aplysia* CNS tissues were briefly ground at 4 °C and centrifuged (Online Methods). The supernatant was collected and fractionated by gel-filtration chromatography. All fractions obtained after gel filtration were analyzed by western blotting using an antibody raised against the *Aplysia* CPEB sequence (Online Methods). These analyses indicate that CPEB extracted from *Aplysia* CNS tissues exists as a high-molecular-weight

oligomer or mixture of oligomers of ten or more units (>80% of the mass of detected protein has an apparent molecular weight of >670 kDa) (Fig. 2 and Supplementary Fig. 1a,b). No monomer was detected by this method. However, consistent with the observations reported previously⁵, trace amounts of oligomers were found in the molecular-weight range expected for a trimer or tetramer. Control experiments done with *Aplysia* α -tubulin (which exists as a monomer as well as a polydispersed polymer *in vivo*; Fig. 2c) and with kinesin heavy chain (which is a dimer in *Aplysia*) showed the expected molecular-weight distribution pattern in our experimental condition. The apparent molecular weight of each fraction was obtained from a calibration curve (Supplementary Fig. 2b).

Recombinant *Aplysia* CPEB was expressed in *Escherichia coli* cells, and the native lysate of these cells was analyzed similarly by chromatography followed by western blotting (Supplementary Fig. 1c). The data also showed a very high apparent molecular weight (>670 kDa) for *Aplysia* CPEB in the soluble form, with a trace amount of smaller oligomers. Moreover, the affinity-purified, soluble *Aplysia* CPEB in phosphate-buffered saline (PBS) containing 2 M urea was analyzed by similar methods, which revealed that large soluble oligomers (larger than decamers) were the dominant forms present in the soluble state. Because the protein in all these three cases eluted near the void volume of the column (670 kDa), determination of the exact apparent molecular weight was not possible, and only a lower molecular-weight cutoff could be determined. Nevertheless, in all three cases more than 80% of the soluble protein was in the form of a high-molecular-weight oligomer, the majority of which had a size of a decamer or larger oligomer.

The similarity in the elution profiles for the native extract from *Aplysia*, the native extract from freshly lysed *E. coli* cells expressing *Aplysia* CPEB (Supplementary Fig. 1c) and the affinity-purified protein suggests that the oligomeric form of *Aplysia* CPEB is likely to be similar to the oligomeric form in the *in vivo* material in *Aplysia* as compared to recombinant material, lending relevance to our biophysical studies *in vitro*.

The soluble form of purified *Aplysia* CPEB in PBS with 2 M urea was further characterized by size-exclusion chromatography followed by light scattering. We found from light-scattering experiments that the oligomeric state of soluble *Aplysia* CPEB in PBS with 2 M urea buffer has 12–20 units and a radius of gyration (R_g) similar to the hydrodynamic radius (R_h), which is around 25 nm. Because the average R_g value is approximately equal to the average R_h value, these oligomers might have a hollow spherical shape under this condition (Supplementary Fig. 2a,b). When the concentration of urea in the PBS buffer was increased to 4 M, these oligomers started to disaggregate into trimer- to monomer-sized particles (Supplementary Fig. 2c) with an extended conformation evident from combined size-exclusion chromatography (SEC) and light-scattering analyses. Although the protein in PBS with 4 M urea buffer eluted at a higher apparent mass than the protein in PBS with 2 M urea, the corresponding light-scattering signal showed a very low molar mass (around monomer to trimer). This might represent some sort of unfolding of the protein, owing to the increase in concentration of urea in the buffer causing an extended conformation for the protein.

Does *Aplysia* CPEB exist in two different conformations?

A relatively simple experiment confirmed that *Aplysia* CPEB indeed forms two distinct conformations with different aggregation and functional capacity. Purified recombinant *Aplysia* CPEB in urea-containing phosphate buffer gradually formed an insoluble aggregate (in 1–2 weeks for a protein solution with a concentration of approximately 1 mg ml⁻¹ or higher in the presence of 2 M urea) and did so more rapidly when the protein concentration was higher or the urea concentration lower. We then tested the ability of the soluble and fiber forms of *Aplysia* CPEB to bind RNA. To probe the RNA binding function, soluble or insoluble *Aplysia* CPEB was incubated with the 3' UTRs of mRNAs coding for N-actin (neuronal actin), which included the CPE elements and were ³²P enriched. The samples were then filtered through a nitrocellulose membrane, which preferentially binds protein but not RNA¹⁹. ³²P-radiolabeled CPE-mutated N-actin RNA was used as one of the controls in this experiment⁴. To eliminate any error due to inherent nonspecific binding of RNA to the insoluble protein, two insoluble *Aplysia* CPEB protein samples were used: the full-length *Aplysia* CPEB mutated at the RNA-binding domain, and the isolated N-terminal domain of *Aplysia* CPEB (prion domain, PRD). We found that, compared with the soluble form, the insoluble full-length *Aplysia* CPEB binds RNA with a significantly greater affinity ($P < 0.05$; Student's *t* test; Fig. 3), which suggests the existence of two functionally different forms of *Aplysia* CPEB *in vitro*. This is notable in light of the *in vivo* data on *Aplysia* CPEB reported previously, which showed that positive function in long-term memory-related synaptic facilitation is associated with the aggregated form of this protein⁵.

Secondary structure of the two forms of *Aplysia* CPEB

The secondary structure of the soluble form of *Aplysia* CPEB was determined by far-UV (180–350 nm) CD. The CD spectrum of the *Aplysia* CPEB protein in PBS with 2 M urea, pH 7.4, showed an α -helix-rich secondary structure (Fig. 4a). Deconvolution of the CD spectrum by using algorithms in DICHROWEB (Contin, Selcon and K2D)^{20,21} showed that the soluble form has 10% or less β -sheet content, 40–50% α -helix content and 30–40% random coil.

The secondary structure of the fiber form of *Aplysia* CPEB was analyzed by FTIR spectroscopy, focusing on the amide I region (1,600–1,700 cm⁻¹). The amide I band, in which β -sheets appear between 1,637 and 1,630 cm⁻¹, has been described to shift below 1,630 cm⁻¹ for β -sheets in amyloid-like structures²². FTIR analysis of the fiber form of *Aplysia* CPEB showed an amide I band at 1,622–1,627 cm⁻¹, which suggests that this form of the protein is predominantly in a β -sheet-rich conformation (Fig. 4b). Manual deconvolution of the spectrum suggests that the aggregated form of *Aplysia* CPEB consists of about 40–50% β -sheets. Synthetic amyloid (A β ₄₂) and BSA (a helix-rich protein) were also analyzed as controls. To further test whether the fiber form of *Aplysia* CPEB has an amyloid-like structure, we used thioflavin-T, a dye that can specifically stain amyloids. Unlike the soluble form, the fiber form of *Aplysia* CPEB showed a bright fluorescence when stained with thioflavin T²³ (Fig. 4c).

X-ray powder diffraction of *Aplysia* CPEB fibers

We further characterized the fibers of purified full-length recombinant *Aplysia* CPEB (expressed in *E. coli*) by X-ray powder diffraction. The fibers formed in PBS were washed three times with water and collected by centrifugation. These fibers were loaded to a loop (as the sample holder) and placed on the path of the X-ray for 1 h. The diffraction pattern of *Aplysia* CPEB showed two well-defined rings at 10.7 Å and 4.7–4.8 Å (Fig. 5a) for the intersheet and interstrand distances, respectively, consistent with an amyloid structure. Yeast prions such as Rnq1p, Sup35p and Ure2p showed similar diffraction patterns for their aggregated fiber form^{24,25}, which supported the idea that *Aplysia* CPEB adopts a β -sheet-rich fiber structure. However, in unusual cases, helical fibers may show prominent diffraction at similar spacing²⁶. Taken together with all the other biophysical analyses and dye-staining experiments on *Aplysia* CPEB fibers, our X-ray diffraction data support a β -sheet-rich (amyloid) secondary structure for these fibers. In contrast, polyethylene glycol-precipitated soluble *Aplysia* CPEB showed no fluorescence with thioflavin T and did not give a diffraction pattern corresponding to an amyloid structure (data not shown). We repeated the experiment by placing the wet fibers in a sealed capillary to avoid any potential drying of the fibers due to the use of a loop as a sample holder. The radial projection of the diffraction pattern obtained from the wet fibers (Fig. 5b) showed a sharp peak at 4.7 Å and slightly broader peak around 10.7–11 Å, indicative of amyloid structures.

Rapid kinetics of fiber formation

The kinetics of fiber formation of the full-length *Aplysia* CPEB were recorded by using real-time fluorescence measurements in the presence of the amyloid-staining dye thioflavin T. To determine whether the conformational change of *Aplysia* CPEB into fibers has a lag phase, the soluble protein in PBS with 2 M urea was directly added to a 40-fold-volume excess of PBS containing thioflavin T, and the fluorescence intensity was measured over time. The data were collected at intervals of 0.1 s. An instantaneous increase in emission intensity following the addition of the protein solution to the PBS-dye solution indicated an apparent lack of a lag phase on the seconds timescale, thus offering evidence for the lack of stable intermediates in the process under the conditions we studied (Fig. 5c). This result is analogous to that for other functional amyloids (for example, Pmel17, condensed peptide hormones or ALF)^{27–30}.

Furthermore, we found that *Aplysia* CPEB fibers, once formed, can be redissolved in 6–8 M urea or 4–6 M guanidine hydrochloride solution. This indicates that the fiber formation of *Aplysia* CPEB is a reversible process *in vitro*.

Limited proteolysis of the fiber form of *Aplysia* CPEB

To identify the fragment involved in the fiber core formation, we carried out limited proteolysis with trypsin and proteinase K followed by MS. We found three fragments missing in the fiber form of *Aplysia* CPEB and present in the soluble form after trypsin digestion and mass spectrometry. Sequencing of these fragments revealed that they are associated with the N terminus of *Aplysia* CPEB (Supplementary Fig. 4 and 5). The SDS-PAGE analysis of the trypsin-digested full-length *Aplysia* CPEB showed a 20- to 25-kDa protease-resistant fragment (Supplementary Fig. 4d). We could not find any protease-

resistant fragment in an analogous experiment carried out using the soluble form of full-length *Aplysia* CPEB. These observations suggest that when *Aplysia* CPEB converts from the soluble form to the fiber form, the conformational change makes the N terminus of the protein resistant to enzymatic digestion. These data are consistent with the model (Fig. 1b) that we derived from the solid-state NMR data indicating that the N terminus of the *Aplysia* CPEB fiber is rigid and that it forms the fiber axis. The sequence of the trypsin-resistant fragment of *Aplysia* CPEB fibers has four intact trypsin-digestion sites (Supplementary Fig. 4f).

Proteinase-K digestion of the PRD and full-length *Aplysia* CPEB fibers showed identical protease-resistant fragments around 7 kDa (Supplementary Fig. 4e). This is consistent with the idea that this fragment might have derived from the N terminus (PRD) of CPEB. To further confirm the position of this fragment, western blot analysis was carried out using an antibody raised against the C terminus of the *Aplysia* CPEB protein. The absence of staining suggests that these peptide fragments belong to the N terminus of the protein. Amino acid analysis showed a glutamine-rich sequence for this fragment, but the exact sequence of the fragment could not be determined from amino acid analysis and MS (data not shown). The details of these experiments are given in Online Methods.

Solid-state NMR analysis of *Aplysia* CPEB

To probe the structure of the fiber form of *Aplysia* CPEB, we used solid-state NMR spectroscopy, including cross-polarization³¹, refocused INEPT (insensitive nuclei enhanced by polarization transfer)³² and direct excitation. The fibers of full-length *Aplysia* CPEB formed in PBS were collected by centrifugation and washed with deionized water to reduce salt-induced sample heating. After the washing, the protein was centrifuged at 14,000 r.p.m. for 30 min to form a compact pellet. This pellet was loaded into the NMR rotor by centrifugation. The cross-polarization experiment selectively detects the static parts of the sample³³, whereas refocused INEPT detects the dynamic portions of the protein. The direct-excitation experiment highlights both the dynamic and the static part of the sample. Solid-state NMR data on full-length *Aplysia* CPEB fibers showed the presence of both static and dynamic domains (Fig. 6a). Other prion proteins like HET-s have also been found to have such dynamical heterogeneity in their amyloid forms^{34–36}. Similarly, the N terminus of Pmel17 fibers has a polymorphism that was revealed by solid-state NMR, which indicates that there may not need to be a unique structure for an amyloid to be functional³⁷.

The PRD also showed similar NMR spectroscopic signatures (indicating the presence of both dynamic and static regions in the protein). However, the refocused INEPT spectrum of the PRD has much less signal associated with the dynamic region of the protein compared to full-length *Aplysia* CPEB (Fig. 6b), which indicates relatively high rigidity at the N terminus of the protein. The 1D spectra showed that *Aplysia* CPEB fibers are dynamically heterogeneous. We investigated the temperature dependence of Hartman-Hahn cross-polarization ¹³C 1D spectra of full-length *Aplysia* CPEB fibers by recording the spectra at 220 K and 260 K. Because the dynamic parts of the fibrils freeze out (or become rigid) at low temperature, we found an increase in signal intensity on the cross-polarization spectra

recorded at a lower temperature (Fig. 6c). This confirms the presence of dynamic parts in the structure of *Aplysia* CPEB fibers.

Aplysia CPEB fibers were characterized by 2D solid-state NMR spectroscopy. The 2D ^{13}C - ^{13}C dipolar assisted rotational resonance (DARR) spectra of the PRD (~200 amino acids at the N terminus; Fig. 7a) was recorded. Previously tabulated average positions are indicated for the $\text{C}\alpha$ - $\text{C}\beta$ and $\text{C}\alpha$ -CO cross-peaks of α -helical, β -sheet and random-coil conformations for serine, alanine, threonine, leucine and glutamine in the 2D DARR (Fig. 7a)³⁸. The assignments of serine, alanine, threonine and leucine were based on their particular chemical shifts, and glutamine could be assigned by the detection of a complete spin system and the fact that this amino acid comprises ~40% of the PRD sequence, thus resulting in the strongest cross-peaks of the entire spectrum (Supplementary Fig. 3b).

The PRD fibers are clearly not composed entirely of β -sheets but also of helices and random-coil elements, on the basis of chemical shifts for serine, alanine and leucine, which are present in the PRD. The presence of two complete and separated spin systems for glutamines, one in a β -sheet conformation, the other in an α -helical conformation, indicates that the PRD has a mixed secondary structure (Supplementary Fig. 3b). This observation is in accordance with the results obtained from the FTIR spectroscopy of *Aplysia* CPEB fibers, which showed about 50–60% β -sheet and the rest 40–50% random-coil and α -helix structures. From the FTIR spectra of the protein it was difficult, however, to distinguish between random-coil and helical structures.

The 2D DARR spectra of full-length *Aplysia* CPEB fibers gave qualitatively the same result as the 2D DARR spectrum of the PRD (Fig. 7 and Supplementary Fig. 3), which suggests that most of the signals in the spectra of the full-length *Aplysia* CPEB spectrum arise from the static PRD, and the RNA-binding domain might be relatively dynamic and, therefore, missing in our spectra. This interpretation is supported by the fact that we observed a marked increase in signal by freezing the full-length *Aplysia* CPEB fibers (Fig. 6c). We hypothesize that in the structure of the full-length *Aplysia* CPEB, the PRD is very rigid, and the C-terminal RNA-binding domain is quite dynamic (Fig. 1b).

The flexible part of *Aplysia* CPEB was further analyzed by using an INEPT-based heteronuclear correlation (HETCOR)³⁹ spectrum in a similar way as was reported for HET-s (residues 218–289) and huPrP (residues 23–144)^{34,35,40}. The HETCOR spectrum of *Aplysia* CPEB (Fig. 7c) showed that most of the amino acids in the dynamic part of the protein are either in α -helical or in random-coil conformation (Supplementary Table 1).

In summary, on the basis of NMR observations, we propose a model for the coordinated polyadenylation of CPE-containing RNAs by the amyloid form of *Aplysia* CPEB (illustrated in Fig. 1b). The RNA-binding assays (Fig. 3) and limited proteolysis experiments (Supplementary Fig. 4) provide additional support for this model.

DISCUSSION

Previous work suggested that *Aplysia* CPEB *in vivo* has prion-like properties and that the aggregated form was the functional state of the protein^{3–5}. These findings motivated us to

ask whether *Aplysia* CPEB exists *in vitro* in multiple conformations with distinct secondary structures. To this end, we have expressed and purified recombinant *Aplysia* CPEB and found the protein to be highly insoluble. Recombinant *Aplysia* CPEB is soluble in phosphate buffer in the presence of 2 M urea, but at lower concentrations of urea the protein promptly forms insoluble aggregates. Biophysical analyses of these two forms confirmed that *Aplysia* CPEB has an α -helix-rich structure in its soluble form and a β -sheet-rich structure in the insoluble aggregated form, with a smaller percentage of helical and random-coil signatures.

The fiber form of *Aplysia* CPEB showed the biophysical signature of an amyloid in FTIR, thioflavin-T staining and X-ray diffraction analyses. Solid-state NMR spectra showed that the PRD of *Aplysia* CPEB is not solely composed of β -sheets but also has helical and random-coil stretches. The alanine, leucine and also some of the glutamine shifts in Figure 7a and Supplementary Figure 3b, in particular, indicate a partial helical content in the PRD fiber of *Aplysia* CPEB. Owing to the lack of site-specific assignments, it is difficult to know whether the structure of *Aplysia* CPEB is polymorphic, comparable to many pathological amyloids, or has a unique structure characteristic of functional prions, similar to the HET-s prion. Prior solid-state NMR studies have shown that the nonprion domains of a number of prion proteins such as HET-s, Ure2p and Rnq1 can be large, sometimes even globular, domains of mixed secondary structure. However, an extended β -sheet conformation was shown to be the dominant secondary structure of the prion domains of all of these proteins^{24,36,41}. Our NMR data for the prion domain of *Aplysia* CPEB revealed the possibility of a new, stable and insoluble β -sheet-rich prion domain with mixed secondary structure containing loops and α -helices. The fact that we detect mixed secondary structure is somewhat of a departure and may result from the use of a larger domain with over 200 amino acids. This is of particular note because another bioinformatics and mutagenesis study from our laboratory predicts that the glutamine-rich regions of *Aplysia* CPEB could have a propensity to form α -helical coiled-coil structures⁴². Whether such coiled coils are the basis of the aggregated state or merely a prelude to β -sheet formation is unclear. Because positive functional amyloid is induced just before the onset of the persistent synaptic facilitation underlying long-term memory, it would clearly be a very dramatic process in the cell that may require regulation of the formation and dismantling of the amyloid. One advantage of having an extensive nonsheet structure, as for example a coiled-coil structure, is that the nonsheet structure could have a regulatory role in these aggregation processes. Moreover, as β -sheet structures are rigid and not accessible to regulation, the nonsheet coiled-coil structure could provide a mechanism for the regulated switching from a soluble to a β -sheet structure and back again. Thus the structure model has the advantage of β -sheet rigidity necessary for a translation-machine model and the flexibility of the coiled coil needed for regulation.

Solid-state NMR experiments on full-length *Aplysia* CPEB indicate that the protein has both very rigid and very dynamic parts: the rigidity of the fibers is apparently associated with the PRD of the protein. In contrast, the C terminus or the RNA-binding domain of the protein is very flexible and could not be observed with solid-state NMR. Dynamical heterogeneity has been reported for other prions as well. This observation leads us to propose a model for the gain of function during aggregation of *Aplysia* CPEB, in which the prion domains of the protein are stacked together to form the fiber axis (Fig. 1). The RNA-binding domains, by

contrast, are dynamic and exposed on the surface of the fiber, free to bind RNA. A similar model was proposed to explain the activity of amyloid-like fibrils of RNase A⁴³. A locally concentrated arrangement of RNA-binding sites could be advantageous for translation at the synapse. It could facilitate localized polyadenylation of multiple RNAs, and it might, therefore, coordinate polyadenylation of a population of inter-related RNAs during synthesis of new proteins that are immediately used at the synapse for the maintenance and stabilization of long-term memory.

METHODS

Methods and any associated references are available in the online version of the paper.

ONLINE METHODS

Plasmids

All plasmid constructs used in this study have been described previously^{3–5}.

The full-length *Aplysia* CPEB and isolated PRD, containing the first 213 amino acids at the N terminus, were expressed and purified as recombinant proteins. They were cloned in frame into the histidine-tag vector pRSETA (Novagen). The His₆-CPEB fusion protein was expressed in *E. coli* and purified by Ni-affinity chromatography under denaturing conditions (Qiagen).

The neuronal actin 3' UTR and mutated CPE-actin plasmids were also described elsewhere⁴. The N-actin 3' UTR was obtained by PCR using the 5' primer 5'-GGGAATTCGTCTGGAGCCACCAACAC-3' and 3' primer 5'-CGGATCCATTTATTAACATTGTATAAAAAATACAGTTGAAC-3'. To mutate the CPE element, the 3' primer was changed to 5'-CGGATCCATTTATTAACATTGTATGGGAAATACAGT-TGAAC-3'. The N-actin 3' UTRs were cloned into the pCR2.1 vector (Invitrogen). Plasmids were linearized by using BamH1 and used for *in vitro* transcription to prepare [³²P]UTP labeling of RNA (Ambion).

The sequence of full-length *Aplysia* CPEB and PRD were expressed in *E. coli*. The bold-faced letters show the beginning and end of native *Aplysia* CPEB sequence.

CPEB:

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSMQAMAVAS
QSPQTVDDQAIKVTDYEDNQEHPSNFEIFRRINALLDNSLEANNVSCSQS
QSQQQQQQTQQQQQQQQQQQQQHLQQVQQQRLLKQQQQQAQRQ
QIQQQLLQQQQQKQQLQQQQQQEQQLQQQLQLQQQLQQQLQHIQKEP
SSHTYTPGPSPELQSVLNANVPLSKSAFNCNNSSSYSGPTPVQSPVTPSP
AASAVTVNSPSYGNFQLFGENAFDSTTPFQSDGTSQSHSRSLANDSDPMVV
MSPGRDSIPLSPTEKILYQNFLSKQAQGENTALPPSPHEIMPLSPLEKKLY
SNLLSKHTQGMRAINSTSPLQTPLTPRSPQEVLYASMPAAQVGESSVIDM
MSRMDLSGRNQADYSGTLAFLDAHNVLRRTTPSSSRSRVMSERSAPSSYF
ANLDPYAIIDRAARLHRNAAVSEASCTWSGHLPPRNHKNPVYSPKVFLG

Purified full-length *Aplysia* CPEB in PBS with 2 M or 4 M urea was analyzed by SEC followed by online static laser light scattering. The soluble protein was filtered (650 μm) and injected onto the SEC column (TSKG6000PW, Tosoh Corp.) equipped with a UV detector and an LS detector (90°). SEC served as a fractionation step based on the size of the particles. The LS detector gave the weight average molecular weight. The radius of gyration (R_g) and hydrodynamic radius (R_h) distribution of the particles were derived from the LS signal.

RNA-binding assay

For the filter-binding assays, nitrocellulose filters (Millipore, HAWP025S0) were preincubated in the RNA binding buffer at room temperature for 30 min and kept in the same buffer until ready to bind RNA. The binding buffer is 50 mM HEPES buffer, pH 7.8, containing RNase inhibitors SUPERaseIn (1 unit per μl ; Ambion), yeast tRNA (1 $\mu\text{g}/\text{ml}$), BSA (1 $\mu\text{g}/\text{ml}$), salmon sperm DNA (1 $\mu\text{g}/\text{ml}$; Ambion) and phosphatase inhibitors (1:100 v/v; Sigma). Soluble oligomers and freshly prepared aggregates (2 μg protein, determined by measuring absorbance at 280 nm) were incubated with *in vitro*-transcribed ^{32}P -labeled wild-type and mutant N-actin RNAs (6,000 μCi) at 25 °C for 30 min. Binding reactions were conducted in siliconized RNase-free 1.5-ml tubes (Ambion). Each treatment had four replicates. The protein-RNA mixture was then transferred onto high-protein-binding nitrocellulose filters (HAWP025S0, Millipore) and placed on a Millipore vacuum filtration manifold apparatus. A brief vacuum was applied, and filters were washed four times with 3 ml binding buffer. The filters were then collected and air dried, and radioactivity was measured by scintillation counter and analyzed by using SigmaPlot.

Circular dichroism and Fourier-transform infrared spectroscopy

CD spectra were recorded with a J-720 CD spectrometer (Jasco) scanning at 20 nm/min, with a bandwidth of 1 nm and data spacing of 0.5 nm, using a 0.1-cm cuvette. Five individual scans were averaged and subtracted from the background.

Infrared spectra of the fibers were recorded by using a PerkinElmer FTIR system purged with nitrogen, employing the diffused reflectance infrared Fourier transform (DRIFT) spectroscopy method. All spectra recorded had a minimum of 512 scans. The background was subtracted from the sample spectrum to obtain the final data.

Thioflavin-T staining and fluorescence measurement

Purified *Aplysia* CPEB protein expressed in *E. coli*, in PBS containing 2 M urea was used for this study. The fibers were produced by adding 0.5–1 mg/ml solution of the protein to a 40 $\times$ volume of PBS containing around 10–25 μM concentration of thioflavin T (Sigma).

The fibers were visualized by using a confocal microscope to measure fluorescence at an excitation maximum of 445 nm and an emission maximum of 485 nm.

Real-time measurements of thioflavin-T fluorescence were collected by using a spectrofluorometer (Fluoromax 3). PBS buffer with dye excited at 425 nm (which is preferable to 445 nm, as it avoids the overlap of Raman scattering with the fluorescence

emission), and the fluorescence emission was monitored at 480 nm as a function of time. Upon the addition of protein solution to the PBS-dye solution, a sudden increase in fluorescence intensity was observed, showing a spontaneous formation of amyloid fibers.

X-ray powder diffraction

Aplysia CPEB fibers were loaded on a loop and were placed in a 1.54-Å-wavelength X-ray beam generated by a rotating anode source operated at 50 kV and 100 mA, coupled with a multilayer focusing optics. The samples were rotated 180° perpendicular to the X-ray beam. Diffraction data were collected for 120 min by using a Raxis IV two-dimensional-imaging plate detector placed 15 cm from the sample. Background diffraction was subtracted from the sample diffraction data to obtain the final diffraction spectra.

Diffraction of wet fibers in a glass capillary was done by loading the fibers in the capillary (0.7 mm) with a drop of water. The diffraction pattern was recorded for 20 min. The background diffraction due to the capillary and water was subtracted to get the resulting diffraction pattern of the fibers.

Limited proteolysis and mass-spectroscopic analysis

Proteolysis of soluble and insoluble *Aplysia* CPEB was carried out by using two different proteases: proteinase K and trypsin. For the proteinase-K treatments, proteinase K (final concentration 1 µg/µl) was added to soluble and insoluble *Aplysia* CPEB and incubated at 37 °C for 30 min. Protease digests were analyzed by SDS-PAGE and MS. Trypsin digestions were carried out overnight. The pellet and soluble fractions were analyzed by SDS PAGE and MS fingerprinting (Columbia University Protein Core Facility).

Digestion and LC-MS or MS analysis of soluble and insoluble *Aplysia* CPEB

Insoluble *Aplysia* CPEB fibrils were suspended in ultrapure water and centrifuged. The supernatant was removed and the wash repeated. The solid material was suspended in 25 mM Tris-HCl, pH 7.5, reduced with DTT and alkylated with iodoacetamide. Trypsin (Roche) was added to the tube, which was incubated at 32 °C for 2 h. This was followed by addition of endoproteinase Asp-N and continued incubation overnight at 32 °C. The soluble form of *Aplysia* CPEB was reduced, alkylated and digested in the same way. After digestion, both preparations were centrifuged to remove any remaining solid material and desalted by using a pipette tip packed with C18 reverse-phase support.

LC-MS and MS analyses were done on a Waters Ultima Q-T hybrid quadrupole time-of-flight mass spectrometer with a nanoelectrospray source. Capillary voltage was set at 1.8 kV and cone voltage at 32 V; collision energy was set according to mass and charge of the ion, from 18 eV to 50 eV. Chromatography was performed on an LC Packings HPLC with a C18 PepMap column, using a linear acetonitrile gradient with flow rate of 200 nl/min. Raw data files were processed by using the MassLynx ProteinLynx software, and .pkl files were submitted for searching at www.matrixscience.com with the Mascot algorithm.

Solid-state NMR

All samples used for solid-state NMR analysis were labeled uniformly with ^{13}C and ^{15}N , as described in a reported procedure^{46,47}. A brief summary of the labeling procedure is as follows: the bacterial culture grown overnight were transferred to fresh LB medium (2 l) containing ampicillin (100 $\mu\text{g/ml}$) and chloramphenicol (34 $\mu\text{g/ml}$) at 37 °C and were shaken for 2 h. When an OD_{600} between 0.6 and 0.8 was reached, the cells were pelleted and washed with 2 l ice-cold M9 buffer and redissolved in 1 l of M9 minimal medium containing 0.5 g of $^{15}\text{NH}_4\text{Cl}$ and 4 g of ^{13}C -labeled glucose (Cambridge Isotope Lab). After 1 h of equilibration at 25 °C, protein expression was induced with 1 mM IPTG, and the cells were harvested after 12 h. The purification procedure was the same as for the unlabeled protein. After the fibers were formed in PBS (pH 7.4) at 4 °C, they were washed with deionized water to remove the salt, which can lead to sample heating and reduce the quality of the NMR spectra. Afterward, the wet fibers were centrifuged into an MAS rotor. The PRD fibers had about 2 mg of protein. The full-length samples measured at 600 MHz and 900 MHz contained around 1 mg of the protein.

CP spectra were done with a ^{13}C field of 50 kHz, adjusting the ^1H RF-field strength to the $\omega_{1\text{H}} = \omega_{13\text{C}} + \omega_{\text{MAS}}$ condition. Unless noted otherwise, we kept the sample at temperatures between 0 and 10 °C. The ^{13}C 1D CP, direct excitation, refocused INEPT and 2D DARR spectra of PRD fibers, as well as the low-temperature cross-polarization ^{13}C 1Ds spectra of full-length *Aplysia* CPEB, were recorded on a Bruker Advance 750 spectrometer using a 4 mm triple-resonance HXY probe operating at 12 kHz MAS. For the 1D spectra, 1,024 acquisitions were added. The 2D DARR spectra were recorded with a mixing time of 50 ms and a spectral width of 50 kHz in both dimensions. The direct dimension was sampled with 1,024 complex points, and 32 acquisitions were added for every 512 points in the indirect dimension. The ^{13}C 1D CP, direct excitation, refocused INEPT and 2D INEPT-HETCOR spectra of full-length *Aplysia* CPEB were recorded on a 600 MHz InfinityPlus spectrometer using a 3.2 mm T3 probe. The 1D spectra were recorded at 20 kHz MAS, adding 1,024 acquisitions. The 2D INEPT-HETCOR was recorded at 11 kHz by using spectral widths of 50 kHz and 20 kHz for the direct ^{13}C and indirect ^1H dimension, respectively. For the direct dimension, 1,024 complex points were recorded, and 384 acquisitions were added for every 128 points in the indirect dimension. The 2D DARR spectrum of full-length *Aplysia* CPEB was recorded on a Bruker Advance II 900 spectrometer using a 3.2 mm E-free probe operating at 20 kHz MAS. The mixing time was 50 ms, and the spectral width was 57 kHz in both dimensions. For the direct dimension, 1,024 complex points were recorded, and 32 acquisitions were added for every 512 points in the indirect dimension.

Data were processed by using Topspin 1.3 (Bruker), and multidimensional spectra were analyzed by using CARA 1.8 (www.cara.nmr.ch).

Acknowledgments

We are extremely grateful to K. Si (Stowers Institute for Medical Research, Kansas City, Missouri, USA) for providing the plasmids used in this study and to S. Jokusch (Columbia University) for helping us with FTIR spectra and real-time fluorescence measurement. We would like to acknowledge and thank J. Lidestri, (Columbia University) and H. Park (Scripps Research Institute) for assisting with X-ray diffraction analyses of the fibers. We are grateful to K. Nakanishi and N. Berova (Columbia University) for their guidance in CD analysis and to M.A.

Gawinowicz (Columbia University) for MS analysis of the protein fragments. This work was supported by the Howard Hughes Medical Institute, US National Institutes of Health (grant MH075026; E.R.K.) and US National Science Foundation (grant MCB0316248; A.E.M.).

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Figure 1. Schematic representation of the neuronal isoform of *Aplysia* CPEB

(a) Domain organization in the neuronal isoform of *Aplysia* CPEB. Pink, prion domain (PRD); blue, RNA-binding domain (RBD), which alone also represents the developmental isoform of *Aplysia* CPEB. The neuronal isoform (expressed only in neurons) and developmental isoform (expressed in other tissues also) of *Aplysia* CPEB differ only in the PRD. Two RNA-recognition motifs (RRM), a glutamine-rich domain (Q rich) and a glutamine- and proline-rich domain (QP) are shown. (b) Model showing the assembly of *Aplysia* CPEB fibers that can efficiently regulate the coordinated polyadenylation of

multiple RNAs in a localized manner. Red, PRD; blue, RBD. The PRDs are stacked together to form the rigid fiber axis, and the RBDs are solvent exposed and oriented outside the fiber axis.

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Figure 2. The soluble form of *Aplysia* CPEB exists as oligomers in *Aplysia* neurons and in *E. coli* expressing *Aplysia* CPEB

(a) Gel-filtration analysis of native total soluble-protein extract from *Aplysia* CNS (red), native extract of recombinant *Aplysia* CPEB purified from *E. coli* (blue) and affinity-purified *Aplysia* CPEB in PBS-2 M urea buffer (green). (b) Western blot analysis using *Aplysia* CPEB antibody of the fractions obtained from gel filtration of *Aplysia* neuronal extracts (red in a). Fractions 21–23 are near the void volume of the column (corresponding to molecular weights >670 kDa). Fractions 31 and 32 correspond to molecular weights in the range of 210–280 kDa, indicative of trace amounts of trimer or tetramer. (c) Control experiment for the western blot analysis of the different fractions, using anti- α -tubulin antibody. As expected, soluble α -tubulin is present in the fractions corresponding to monomer through very high-molecular-weight oligomers.

Figure 3. RNA-binding assay

RNA-binding efficiency of full-length *Aplysia* CPEB (FL CPEB; red), isolated prion domain (PRD; cyan) and full-length *Aplysia* CPEB mutated at the RNA-binding domain (FL CPEB mRBD; brown) bound to a ^{32}P -labeled N-actin RNA 3' UTR probe. As shown, full-length *Aplysia* CPEB in its soluble form (pink) displayed minimal RNA binding, and negative-control N-actin RNA mutated at the CPE element allowed roughly the same binding as that of mutated protein to wild-type N-actin RNA. The conformational change of *Aplysia* CPEB resulted in a significant increase in binding affinity toward its target mRNAs, compared to that of the soluble form ($P < 0.05$; Student's t test; $n = 6$). Error bars, s.e.m.

Figure 4. Secondary-structure analysis of two conformational forms of full-length *Aplysia* CPEB (a) CD spectrum of purified recombinant, soluble full-length *Aplysia* CPEB (blue) in PBS–2 M urea buffer, indicating a helix-rich secondary structure. For comparison, the CD spectrum of an α -helix-rich protein, BSA in PBS with 2 M urea (red), is shown. (b) FTIR analysis of fibers formed from full-length recombinant *Aplysia* CPEB. The spectrum of *Aplysia* CPEB (red) is compared with those of helix-rich protein BSA (blue) and β -sheet-rich protein A β_{42} amyloid (green). The amide I band of full-length *Aplysia* CPEB at 1,622–1,627 cm^{-1} indicates a β -sheet-enriched secondary structure comparable with the spectrum of A β_{42} . α -helix-rich proteins are predicted to have an amide I band peak maximum at 1,656 cm^{-1} , as in the case of BSA. As shown, this peak (1,656 cm^{-1}) is absent in the spectra of *Aplysia* CPEB and A β_{42} . (c) Confocal projection image showing thioflavin-T staining of the fiber form of full-length *Aplysia* CPEB, indicative of an amyloid-like structure. The soluble form showed only background fluorescence.

Figure 5. X-ray powder diffraction analyses of *Aplysia* CPEB fibers

(a) X-ray powder diffraction pattern of nonoriented full-length *Aplysia* CPEB fibers. The graph is generated from the X-ray diffraction pattern by using ImageJ⁴⁴. (b) Radial projection of the X-ray diffraction pattern of wet *Aplysia* CPEB fibers in a sealed glass capillary. The resultant diffraction pattern subtracted from the background diffraction of the capillary and water is shown. (c) Kinetics of fiber formation of *Aplysia* CPEB, showing no lag phase for the fiber formation of full-length *Aplysia* CPEB upon addition of soluble full-length *Aplysia* CPEB (1 mg ml^{-1}) in PBS containing 2 M urea to a 40 $\times$ excess of PBS containing thioflavin T.

Figure 6. 1D solid-state NMR spectra of the fiber form of full-length *Aplysia* CPEB and the isolated prion domain, indicating dynamic RNA-binding domains and static prion domains

(a) ^{13}C 1D spectra of recombinant full-length *Aplysia* CPEB in hydrated insoluble (fiber) form recorded at a ^1H frequency of 600 MHz and 13 kHz MAS. The initial ^{13}C magnetization was generated by using either a simple 90° ^{13}C pulse (direct excitation, DE), Hartman-Hahn cross-polarization (CP) or a refocused INEPT. (b) Corresponding ^{13}C 1D spectra of the isolated prion domain of *Aplysia* CPEB (PRD) recorded at 750 MHz ^1H frequencies and 12 kHz MAS. (c) Temperature dependence of ^{13}C cross-polarization 1Ds of full-length *Aplysia* CPEB fiber, recorded at 750 MHz ^1H frequency, 12 kHz MAS using a set temperature of 220 K and 260 K. The dynamic parts of the fibril, predominantly located in the RNA-binding domain, freeze out at low temperatures and become visible using solid-state NMR pulse sequences (CP).

Figure 7. 2D solid-state NMR spectra on the N-terminal prion-like domain of *Aplysia* CPEB
(a) ^{13}C - ^{13}C DARR spectrum of the PRD recorded at a ^1H frequency of 750 MHz, 12 kHz MAS and mixing time of 50 ms. The average $\text{C}\alpha$ - $\text{C}\beta$ cross-peak positions corresponding to an α -helical (red), extended β -sheet (orange) and random-coil (blue) conformation for the amino acids threonine, serine, glutamine and alanine, as well as the corresponding $\text{C}\alpha$ -CO cross-peaks for threonine and glutamine, are marked with ellipsoids. The full ^{13}C connectivity of glutamine in α -helical versus β -sheet conformations are also shown with red and orange bars, respectively. **(b)** Overlay of ^{13}C - ^{13}C DARR spectrum of full-length *Aplysia* CPEB (recorded at a ^1H frequency of 900 MHz, 20 kHz MAS and a mixing time of 50 ms) with the DARR spectrum of the isolated prion domain shown in **a**. **(c)** ^1H - ^{13}C INEPT-HETCOR spectra of full-length *Aplysia* CPEB fiber. Tentative amino acid assignments are indicated.