Structural determinants of conformationally selective, prion-binding aptamers

Natalie M Sayer, Matthew Cubin, Alexandre Rhie, Marc Bullock, Abdessamad Tahiri-Alaoui, and William James

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.

Running Title: prion-binding aptamers
Summary

We have recently described the isolation of 2'Fluoro pyrimidine-substituted RNA aptamers that bind selectively to disease-associated β-sheet-rich forms of the prion protein, PrP, from a number of mammalian species. These aptamers inhibit the accumulation of protease-resistant forms of PrP in a prion-seeded, in vitro conversion assay. Here we identify the minimal portions of two of these aptamers that retain binding specificity. We determine their secondary structures by a combination of modeling and solution probing. Finally, we identify an internal site for biotinylation of a minimized, synthetic aptamer and use the resultant reagent in the detection of abnormal forms of PrP in vitro.
Introduction

Transmissible spongiform encephalopathies (TSEs) involve spongiform degeneration and astrocyte gliosis in the central nervous system leading to dementia and death (1). They can have genetic, sporadic or infectious aetiologies, exemplified by Fatal Familial Insomnia, sporadic Creutzfeldt-Jakob Disease (CJD) and vCJD in humans, respectively. They all involve the modification of prion precursor protein PrPc, which is constitutively expressed in mammalian cells, particularly neurons, as a GPI-anchored plasma membrane glycoprotein. During many TSE diseases, infectivity is found to be associated with amyloid deposits of PrP, in a form referred to as PrPSc (2), and lack of a gene for PrP renders animals insusceptible to Prion diseases (3,4). These and related observations support the prion theory of TSE aetiology, in which self-templated conformational changes in PrP are necessary and sufficient for disease progression and transmission (5). The encipherment of the pathological properties of TSEs within the conformation of PrP is not fully understood, but whereas the membrane-proximal domain of PrPc is mainly alpha helical in structure, PrPSc is dominated by beta sheets (6). In contrast to PrPc, PrPSc is insoluble in nondenaturing conditions and is partially resistant to proteases (7).

Diagnostic tests for the in vivo identification of prions are not yet ideal. Disease cannot be confirmed until clinical signs appear, or even until a post mortem of brain tissue shows spongiform changes and prion accumulation using immunohistochemical methods. Only recently has it been possible to isolate antibodies that are able to differentiate between PrPc and PrPSc (8), so current methods rely on a pre-treatment with protease, which may obscure the presence of relevant, protease-sensitive
conformations. The most promising of diagnostic tests have been using fluorescence, such as Multi-spectral ultraviolet fluorescence (MUFS) looking at specific fluorescence of proteins when excited by UV radiation (9), and fluorescence correlation spectroscopy (FCS), which recognizes single fluorescent molecules in solution as they pass between exciting laser beam and objective of confocal microscope (10). Another interesting approach by Safar et al is that of a conformation dependent immunoassay, this uniquely quantifies PrP isoforms by the binding pattern of different antibodies to both denatured and native forms (11). There has been some evidence that PrP\textsuperscript{sc} can be detected in the blood of infected rodents (12,13), as well as in the urine of scrapie-infected hamsters (14). The amount of PrP\textsuperscript{sc} outside the CNS is very small compared to that in the brain, so it might be necessary to amplify the small amount there using, for example, the cyclic PrP\textsuperscript{sc} amplification process (15). For a review of these and other diagnostic assays see (16).

Given the limitations of antibodies, aptamers provide an alternative approach to the ligand-based detection of PrP\textsuperscript{sc}. Aptamers are nucleic acids isolated \textit{in vitro} for their ability to bind a molecule of interest by the SELEX procedure (17). This involves cycles of binding and amplification starting from about 10\textsuperscript{14} different nucleic acid molecules, which eventually results in the isolation of a few sequences that have good affinity for targets such as CD4 (18), Streptavidin (19) and HIV-1 gp120 (20,21). Protein binding by aptamers results mainly from hydrogen bonds and electrostatic forces, which means that the best targets are often basic, positively charged molecules. Aptamers can have a sub-nanomolar affinity and a high specificity (for a review see (22)). Because of the number of different potential ligands that can be screened by the SELEX process,
aptamers have often been raised against molecules when other approaches have failed. They have many advantages over antibodies in that they are non-immunogenic, smaller, usually of a better affinity and can be chemically synthesized. This, together with their contrasting physico-chemical properties to antibodies, makes aptamers potentially useful reagents for the detection of previously overlooked conformations of PrP. We have recently described the isolation of 2′F-pyrimidine-substituted RNA aptamers that were selected from randomized libraries using infectious prions as the target material (23). One of these aptamers, SAF-93 was shown to bind to two regions of PrP: (i) a conformationally insensitive, diffuse region lying between residues 23 and 110 that binds non-specifically to nucleic acids; (ii) a site lying between residues 110 and 230. The latter site was occluded in the normal alpha helix-rich conformation of the protein but exposed in the disease-associated beta sheet-rich forms and was not bound by control nucleic acids. SAF-93 also inhibited the conversion of α helix-rich to β sheet-rich forms in a prion-nucleated, in vitro conversion assay (23). SAF-93 is therefore an interesting ligand for PrP, and may reveal conformations of PrP not yet identified.

Before the properties of this and another aptamer, SAF-76 also described in this paper, could be exploited, more information was required about the structural features essential for their binding to prions. In this paper, we present the results of experiments designed to facilitate the use of prion-binding aptamers as molecular reagents for the investigation of prion diseases. Firstly, we determined the solution structure of the aptamers and mapped the binding motifs onto the determined structure. We then characterized the minimal structural features that were responsible for specific binding and designed a novel method for determining a residue within the essential structure of
the best aptamer that could be modified without affecting its affinity for PrP. With this information, we went on to synthesize modified aptamers for a range of analytical and diagnostic applications.
Experimental Procedures

Protein preparation and refolding

Full length bovine PrP, corresponding to residues 23-230 (human numbering) was cloned, expressed, purified as described before (23). Briefly, the protein was expressed in E. coli and purified from inclusion bodies by Ni-NTA affinity chromatography and RP-HPLC. The purified PrP was refolded into native α-helical conformation or β-oligomeric isoform following the protocol described by (24). Finally, monomeric α-helical rich form or β-oligomer isoform of PrP were purified by high-performance size-exclusion chromatography (23). The structure was checked using circular dichroism (CD) as previously described (23).

In vitro transcription

Transcription of 2’F-pyrimidine-containing RNA was carried out largely as described by (25). Briefly, transcription by T7 RNA polymerase (New England Biolabs) was incubated at 37 °C for at least 6 h in 40 mM Tris pH 8.1, 6 mM magnesium chloride, 1 mM spermidine, 5 mM DTT and 1 mM NTPs. The products were then treated for 30 min at 37 °C with 50 U DNase 1 (Roche) in 150 mM sodium acetate pH 5.2, 10 mM magnesium chloride. The reaction was stopped by phenol extraction, and the transcript recovered by ethanol-precipitation.

32P-end labeling of RNA

For 5’ labeling, the terminal 5’ phosphate was removed using bacterial alkaline phosphatase (New England Biolabs) and replaced with 32P-phosphate from [γ-32P]ATP using T4 polynucleotide kinase (Roche). Labeling at the 3’end was achieved by ligating
[\textsuperscript{32}P]pCp to the 3’ end of the aptamer using T4 RNA ligase (New England Biolabs). Ligation was carried out for 2 h at 37 °C. The labeled RNAs were electrophoresed on 12% polyacrylamide-8M urea gels, visualized by autoradiography, and recovered by passive elution from gel slices.

**Identification of the core binding sequence using boundary experiments**

A library of nested deletion fragments was generated from either 3’ or 5’ end-labeled aptamer by mild alkaline hydrolysis (26), and partitioned into PrP-binding and non-binding fractions using StrataClean™ (Stratagene, CA), as described before (20). The products were analysed by denaturing 18% PAGE.

**Enzymatic probing and footprinting**

\textsuperscript{32}P end-labeled RNA was digested in 1xHMK, pH 7.2, buffer containing 1 µg tRNA at 20 °C for 5 min with either nuclease V1 (Pierce, Milwaukee, WI; 5 x 10\textsuperscript{-3} U), or S1 (Pharmacia-Amersham, UK 0.05 U). Footprinting was achieved by incubating an identical aptamer preparation with prion protein for 1 h at 25 °C and then subjecting the mixture to appropriate nuclease digestion. The digestions were terminated by phenol extraction. The RNA was precipitated using ethanol, then dissolved in formamide buffer, and analysed by denaturing PAGE (see below).

**Urea-denaturing polyacrylamide gel electrophoresis (PAGE) of \textsuperscript{32}P-labeled RNA**

All samples for truncation and footprinting analysis were heated to 95°C for 3 min in formamide loading buffer and run on 40-cm long 18% or 20% polyacrylamide, 8M urea gels at 25 W for 3 h. A partial alkaline hydrolysate of the RNA along with a G-residue ladder was run alongside analysis samples to aid sequence identification. The G-
residue ladder was generated by digestion of 50,000 c.p.m. (Cerenkov) denatured RNA at 55 °C in 10 µL 20 mM sodium citrate, 1 mM EDTA, 7M urea, pH 4.6, with RNase T1 (Amersham Pharmacia Biotech 5 x 10^{-3} U).

**Binding affinity of aptamers**

End-labeled aptamer (5000 c.p.m. Cerenkov) was heat-denatured at 95 °C for 2 min in water, cooled to room temperature and refolded in 1x AMNK buffer (20 mM sodium acetate pH 5.2, 100 mM sodium chloride, 50 mM potassium chloride, 5 mM magnesium chloride, 0.04% NP40) for 10 min. After refolding, the aptamer was incubated with different concentrations of prion protein in the presence of 1µg tRNA in 30 µL for 1 h at 25 °C. The bound RNA was then separated from unbound using 0.1 vol StrataClean™ resin, centrifuged at 13,000 r.p.m. for 3 min, and the supernatant (containing unbound RNA) was removed. The resin was washed with 30 µL 1x AMNK buffer and again centrifuged (the wash was pooled with the unbound RNA). The resin (complexed with PrP-bound RNA) was resuspended in 60 µL 1x AMNK buffer and the amounts of radioactivity corresponding to PrP-bound and unbound RNA were measured for each concentration of protein. The data were fitted using non-linear curve fitting to a hyperbolic equation by Graph Pad Prism.

**Identification of sites at which incorporation of biotin does not interfere with PrP binding**

Transcription of aptamer was done as described above, with the addition of 2 µM biotin-16-dUTP (Roche). After completion, the transcription was dialysed against water for 3 h instead of phenol extraction to remove non-incorporated 5-allyl biotinyl UTP. In order to isolate molecules that could bind a single Streptavidin tetramer, the dephosphorylated,
biotinylated RNA was incubated for 30 min with 30 µL Streptavidin (2 mg/mL). The complex was then run on an 8% Tris-borate native polyacrylamide gel and the band corresponding to aptamer bound to one tetramer of Streptavidin was cut out, passively eluted from the gel, and ethanol-precipitated. The gel-purified and biotinylated RNA was then 5’end radio-labeled as described above.

The double-labeled RNA was ethanol-precipitated and complexed with Streptavidin as before. The complex was then incubated with 40 µL of beta form PrP$_{90-230}$ (1 mg/mL) for 30 min at 25 °C. The ternary complex (PrP-aptamer- Streptavidin) was then partitioned from the binary complex (aptamer-Streptavidin) using StrataClean™ resin (to which Streptavidin does not bind). RNA was recovered from both fractions, partially hydrolysed using alkali and analysed by urea-denaturing PAGE, as described above, along with a transcript without biotin incorporated as control.

**Slot Blots using chemically synthesized biotinylated aptamer**

30 pmol full-length PrP was bound to PVDF membrane (equilibrated in 0.8 % (v/v) acetic acid, 350 mM β alanine) by applying a vacuum. The membrane with protein attached was then washed with TBS Tween and 10 mM magnesium chloride, and then prehybridized in the same buffer with the addition of 5 % milk powder and 20 µg/mL tRNA for 1 h. The membrane was then incubated overnight in 20 pmol SAF-93$_{(1-34, 35bU, 36-60)}$, (chemically synthesized and biotin derivitized at position 35) which had been refolded in 1 X HMNK and added to 20 mL TBS-Tween, 10 mM magnesium chloride, 5% milk powder 20 µg/mL tRNA. The membrane was blocked again for 1h, washed and incubated in presence of Streptavidin alkaline phosphatase (0.25 µg /mL) (Sigma) for 30
min. Then washed and developed using ECF substrate as described before. All incubations were carried out at 25 °C.
Results

Primary and secondary structure determination of SAF-76 and SAF93.

In order to elucidate their secondary structures, SAF-76 and SAF-93 were transcribed in vitro, purified, labeled at the 5’ end with $^{32}$P, and re-folded. They were then subjected to limiting digestion using the structure-sensitive nucleases S1 (preference for single-stranded regions) and V1 (preference for double-stranded regions) (27) and analysed using denaturing PAGE, as described (see methods). The results (see supplementary data) were used to constrain the MFOLD helix-prediction algorithm (28) and to eliminate the sub-optimal structures that were inconsistent with experimentally determined structural features. The most empirically compliant structures are illustrated in Figures 1A and 1B. It must be noted that RNA structures are often dynamic (29). Aptamers, which have been selected for their ability to bind to a target protein with good affinity, may not always be fully structured in the absence of protein. For this reason, even though multiple repetitions of these experiments gave entirely consistent results, we cannot be categorical that these putative secondary structures are as well-determined as, say, those of tRNAs.

With these caveats, the aptamers appear to fold into two and three helical domains, respectively, separated by extended, non-helical regions. That is, SAF-76 appears to comprise two domains consisting of helices 1, 2 & 3 and 4, 5 & 6, respectively and SAF-93 to comprise three domains consisting of helices 1, of 2, 3 & 4 and 5, respectively. It seemed very possible that only one of these domains in each case was actually necessary for interaction with PrP, particularly as the aptamers are relatively long (117 and 116 nt. respectively). Accordingly, we determined the 3’ boundary of the PrP-
binding regions of each by separating a partial hydrolysate of 5'-labelled full-length aptamer into PrP-binding and PrP-non-binding fractions, and analysing each by urea-denaturing PAGE (see Figures 1C and 1D).

In each case, an approximate 3' boundary could be identified as the point at which the intensity of fragments in the lane of non-binding RNA became greater than that in the lane of PrP-binding RNA, as indicated in Figure 1. Again, it should be noted that even very short fragments retain some binding to PrP, probably through the non-specific interaction with the N-terminal tail of the protein. This results in a slightly blurring of the boundary between the essential and inessential regions of aptamer. Nevertheless, in both cases, it seemed possible to eliminate a substantial 3' portion of the aptamer without loss of PrP-binding. When the 3' boundaries were placed on the secondary structure previously determined, a paradox immediately presented itself in both cases.

In SAF-76, helix 1, and in SAF-93, helix 2 were split by the 3' boundary. That is, in each case, only one half of a predicted helix remained in the 3' truncated, PrP-binding aptamer.

Three explanations seemed possible. Firstly, the actual secondary structures might be very different from those presented. This could result from a large number of non-canonical, tertiary interactions stabilizing much weaker canonical helices than had been considered by the MFOLD algorithm. Second, the truncated aptamer might still bind to PrP but with much reduced affinity, as a result of the loss of one helix. Thirdly, the truncated aptamer might fold into an altogether different structure from its full-length parent. To resolve these possibilities, we used the primary and secondary structure.
information to generate defined, truncated aptamers, and analysed both their affinity for PrP and their secondary structures.

**Structure and affinity of truncated aptamers.**

The dsDNA template used to transcribe the parental aptamers *in vitro* was amplified using the standard 5’ SELEX primer together with a 3’ primer corresponding to the 3’ boundary determined above. The parental aptamers and their truncated derivatives are hereinafter named to indicate the nucleotides retained from the parent.

These truncated derivatives were then analysed by enzymatic probing as described previously. Again the experiments were repeated a number of times to ensure the cleavages shown were consistent. SAF-76\(_{(1-38)}\) showed anomalous nuclease sensitivity when compared with its parent, SAF-76\(_{(1-117)}\) (see Figure 2A). Nucleotides that had formerly appeared to be single-stranded were now sensitive to nuclease V1, while those previously showing signs of double-strandedness were now sensitive to nuclease S1, making the parent-like structure shown in Figure 2A untenable. When we constrained the modelling of SAF-76\(_{(1-38)}\) by MFOLD with the new data, the structure shown in Figure 2B emerged. Intriguingly, this contains secondary structural features found within the 5’ region of SAF-93, with which it shows some sequence homology. When we compared the affinity of the truncated aptamer with its parent (see Figure 2C), it appeared that removal of the 3’ portion reduced the affinity of SAF-76 for \(\alpha\)-form PrP but maintained its affinity for \(\beta\)-form PrP. This is consistent with the idea that binding to the \(\alpha\)-form is dominated by non-specific nucleic acid interactions in the unstructured N-terminus of PrP, which would lessen as the length of the RNA decreased. Although the conformational specificity of SAF-76\(_{(1-38)}\) to \(\beta\)-oligomeric form PrP was improved
compared with the full-length aptamer, its affinity was still weaker than that of aptamer SAF-93.

We constructed three deletion mutants of SAF-93. The first, SAF-93\textsubscript{(1-60)}, was based on the 3' boundary determined above. The second, SAF-93\textsubscript{(3G, 28-60)}, was, in addition, truncated at the 5', in accordance with indications from a 5' boundary experiment (data not shown), thereby removing helix 1. The third, SAF-93\textsubscript{(1-33, 43-45, 48-51)}, was designed to preserve helix 1, shorten helix 3, and remove sequences corresponding to the “half-helix” 2. Nuclease sensitivity experiments, together with computer modelling, indicated that the secondary structure of each of these truncated aptamers was as anticipated (Figure 2D). Interestingly, nucleotides 31-32 of SAF-93\textsubscript{(1-60)}, which had appeared to be in helix 2 of the full-length SAF-93\textsubscript{(1-116)}, were still sensitive to nuclease V1 in the truncated version, in which helix 2 could not form. This is strong evidence for stable, non-canonical interactions between this region and some other portion within the 5' half of SAF-93.

When we measured the affinity of the truncated versions of SAF-93 for both conformations of PrP, we found that removal of the 3' half to generate SAF-93\textsubscript{(1-60)} had little effect on the binding to the α-form while slightly improving the affinity for the β-oligomeric form (see Figure 2E). The shortening of helix 3 and removal of region 2 in SAF-93\textsubscript{(1-33, 43-45, 48-51)}, reduced the binding affinity for the β- oligomeric form of PrP.

However, destruction of helix 1 in SAF-93\textsubscript{(3G, 28-60)}, and disruption of region 2, comprising presumptively tertiary structure, effectively destroyed the specificity of binding to β-form PrP. These results correlate with the footprinting studies on SAF-93\textsubscript{(1-}. 
which showed that much of the sequence was protected by binding to β-form PrP (see supplementary data).

Taken together, the results show that SAF-93(1-60) defines a minimal, conformationally-selective aptamer for PrP and comprises two helices bounding a region of tertiary structure.

**Generation of an internally derivitized aptamer for PrP detection**

One consequence of the minimization procedure, above, was that relatively little of SAF-93(1-60) was free to interact with bulky molecules such as Streptavidin once it was bound to β-form PrP, as indicated by the footprinting results (see supplementary data).

This was disadvantageous in many potential applications of aptamer technology. Indeed, introduction of a biotin at the 3’ end – the most convenient position for site-specific, post-transcriptional incorporation – resulted in a ligand which, when complexed with Streptavidin, had very poor PrP-binding characteristics (data not shown).

Consequently, we undertook a systematic search for internal uracils that could be so derivitized without detriment.

We exploited the fact that all the uracils in SAF-93(1-60) were part of the non-hydrolysable nucleotide, 2’F, 2’ deoxyuridine. This means that, in the unmodified SAF-93(1-60), a partial alkaline hydrolysate of 5’ labeled aptamer gives a purine ladder, and bands corresponding to “U” are absent. On the other hand, biotinylated uracil was associated with 2’ OH 5-allyl-biotinyl uridine, which is readily cleaved by alkali, producing an additional band at any U position into which it is incorporated. We spiked transcription of SAF-93(1-60) with 2µM 5-allyl-biotinyl UTP in order to produce an average
incorporation rate of one biotinylated uracil per molecule, presumably at random, at the
nine available positions. We then complexed the partially biotinylated RNA with
Streptavidin (SA), and purified the RNA that was bound to one SA tetramer, from that
which bound none, two or more molecules of SA. We then incubated the SA-aptamer
complex with PrP and partitioned the PrP-binding complexes using the resin
StrataClean™, which fortuitously binds PrP but not SA. We subjected the PrP-binding
and non-binding complexes to partial alkaline hydrolysis and analysed them on
denaturing PAGE (see Figures 3A and 3B).

The results show that the most 5’-proximal uracil that can be derivitized with biotin-
streptavidin while retaining its PrP-binding properties is U35. In the 5’ region of U35, the
hydrolysis ladder is identical in both the biotin-SA-PrP-binding sample and the control
sample. However, in the 3’ end of U35, there is an additional cleavage event in the
biotin-SA-PrP-binding sample, the bands migrate more slowly (by approximately one nt.
equivalent) and are less distinct than in the control sample. This is consistent with the
incorporation of an additional 487 Da (corresponding to allyl-biotin) and the replacement
of 2’F with 2’OH at position 35. Because of the resolution of the gel, it is not possible to
exclude the possibility that uracils 3’ to U35 could also be derivitized in this way without
loss of function.

To test whether our interpretation of this experiment was correct, we chemically
synthesized an aptamer corresponding to the sequence identified above, namely SAF-
93(1-34, 35bioU, 36-60) and determined its affinity for the two isoforms of PrP when complexed
with Streptavidin. The results show that, as anticipated, the affinity of the aptamer for β-
form PrP was the same in the absence as in the presence of Streptavidin: Kd = 8.4±1.4 nM and 6.8±2.9 nM, respectively (see Figure 4 A).

This aptamer was then used to probe slot blots of PrP in either the β- oligomeric or α- monomeric conformation (Figure 4B). The aptamer detected the β form much more effectively than the α form and did not bind to an equivalent amount of human serum albumen.
Discussion

We have recently described aptamers that bind a region of PrP that is intimately involved in the conformational shifts that occur in the generation of prions, and can inhibit conversion in cell-free assays systems (23). Although the original aptamers had many promising features, for example, their resistance to degradation conferred by 2'F substitution, they were not ideally suited to analytical or therapeutic applications because of their length and complexity. Accordingly, we have used a range of experimental and computational tools to define the essential functional structures of two prion-binding aptamers.

By analysing the functional primary and secondary structure of the original 116 nt. long aptamer using a range of techniques, we have been able to preserve its conformational specificity while reducing it to a size suitable for chemical synthesis. Moreover, using a new method for screening internal modifications, we have been able to derivitize it with biotin to generate a ligand that can readily be applied in multiple detection formats. The synthetic aptamer described here (SAF-93(1-34, 35bioU, 36-60)) is a novel and promising tool for the investigation of PrP isoforms in vivo.

For example, it could be used as a detection reagent in slot or dot-blotting applications, as shown here. Specifically, one could envisage applying multiple samples of blood, cerebro-spinal fluid, brain homogenate, or other biological materials in parallel to a PVDF or similar support membrane. SAF-93(1-34, 35bioU, 36-60) could then be used to develop the membrane, followed by detection using enzyme-conjugated Streptavidin and chemoluminescent, chemofluorescent or chromomogenic imaging. Perhaps more
conveniently, high-throughput, plate format assays could be developed, in which aptamer-derivitized assay plates are used to capture abnormal PrP from biological samples. Detection of the captured PrP could again by through the use of SAF-93\(_{1-34, 35\text{bioU}, 36-60}\) and any of the proprietary Streptavidin-based systems.

More biologically informative uses include the replacement of anti-PrP antibodies with SAF-93\(_{1-34, 35\text{bioU}, 36-60}\) in histochemical analysis of biopsy and post-mortem samples of experimentally infected model animals or suspect cases. Certain investigations that are not possible using currently available antibodies might become possible using the aptamer. For example, the specificity of SAF-93\(_{1-34, 35\text{bioU}, 36-60}\) for abnormally folded PrP does not depend on the resistance of the protein to proteinase K. This means that putative forms of PrP that are abnormally folded and either infectious or pathogenic but not fully resistant to PK could be sought directly. Moreover, because the selective reactivity of abnormally folded PrP for aptamer is expressed under native conditions, and doesn’t require the presence of denaturants, as is commonly the case with antibody-based systems, the aptamer approach opens the way for a simpler exploration of the appearance of infectious material in vivo under conditions in which classic PrP\(^{Sc}\) is not presently detectable.
References


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Figure legends:

Figure 1. Minimal primary and secondary structure of PrP-binding aptamers.

Aptamers SAF-76 (panels A and C) and SAF-93 (panels B and D) were subjected to secondary structure analysis and 3' boundary determination. To determine secondary structures (panels A and B), 5' labeled RNA was probed with the structure-sensitive nuclease, V1, S1 and T1 and analysed by denaturing PAGE (see supplementary data). The results were used to constrain the MFOLD helix-prediction algorithm (28) and to eliminate the sub-optimal structures that were inconsistent with experimentally determined structural features. The most empirically compliant structures are illustrated, and residues found to be cut with V1 are shown in blue, and those cut by S1 in red. To determine the 3' boundary of the minimal PrP-binding sequence (panels C and D), a partial hydrolysate of 5'-labelled full-length aptamer was partitioned into PrP-binding and PrP-non-binding fractions, and was analysed by denaturing PAGE together with a T1 digest (G-ladder) and an sample of unfractionated hydrolysate (purine ladder). Arrows indicate the 3' boundary inferred from the gel and relate this to the corresponding portion of the appropriate secondary structure.

Figure 2. Structures and binding properties of truncated PrP aptamers.

Truncated aptamers were designed using the information from the boundary experiments (Figure 2). Subscript figures indicate the parental aptamer nucleotides retained in the truncated aptamer. Secondary structures (panels A, B and D) were determined by a combination of enzymatic probing and computer modeling as described. Residues sensitive to V1 are shown in blue and to S1 in red. The affinity for both β and α forms of PrP of parental and truncated derivatives of aptamer SAF-76
(panel C) and SAF-93 (panel E) was determined by equilibrium binding, as described in Methods. Briefly, 5' labeled aptamer was incubated equilibrated with a range of concentrations of PrP and left and protein-bound RNA was separated from unbound using StrataClean™.

Figure 3: PrP-footprinting and internal biotinylation of SAF-93(1-60)

The secondary structure prediction consistent with both Enzymatic probing and footprinting results is presented for SAF 93(1-60). Nucleotides showing an PrP footprint are in red. Experiments were then carried out on this aptamer to find a residue that could be substituted with a biotin without affecting affinity. All the uracils and cytosines in SAF-93(1-60) are modified with Fluoro at the 2’ hydroxyl. This means that, in the parent SAF-93(1-60), a partial alkaline hydrolysate of 5’ labeled aptamer gives a purine ladder, and bands corresponding to “U” are absent. Therefore if a biotinylated uracil is incorporated with 2’ OH this will be readily cleaved by alkali, producing an additional band at any U position into which it is incorporated. SAF-93(1-60) was transcribed under conditions where only one biotinylated uracil was incorporated, 5’ radiolabeled, incubated with Streptavidin (SA) and the complex of RNA binding one tetramer isolated. The SA-aptamer complex was then incubated with PrP and the ternary complexes comprising SA-aptamer-PrP were partitioned from the remaining PrP-unbound SA-aptamer using StrataClean™. Panel A shows denaturing PAGE analysis of a partial alkaline hydrolysate of control SAF-93(1-60) along with the biotinylated SAF-93(1-60) isolated from the SA-aptamer-PrP complexes. Panel C shows a magnification of the same gels. Lines indicate corresponding residues in the aptamer. The position of U35, at which a biotin appears to be incorporated in PrP-binding aptamers, is indicated with a
blue asterisk. Panel C shows a representation of the secondary structure of SAF-93\textsubscript{(1-60)} in which U35 is highlighted in blue and those residues found to protected by PrP in footprinting experiments (see supplementary data) are shown in red.

**Figure 4** The PrP-binding properties of a synthetic internally biotinylated, truncated derivative of aptamer SAF-93

Panel A. The Kd of SAF-93\textsubscript{(1-34, 35bU, 36-60)} for the β form of PrP in the presence (■) and absence (▲) of Streptavidin was determined by equilibrium binding assays to be 8.4±1.4 nM and 6.8±2.9 nM, respectively. Panel B shows a slot blot assay, in which 30 pmol of beta-form and alpha-form PrP and of human serum albumen (HSA) were applied in duplicate to a PVDF membrane and them probed with SAF-93\textsubscript{(1-34, 35bU, 36-60)}. The blots were processed with Streptavidin-alkaline phosphatase and ECF substrate, and imaged using a STORM 840 system.
Supplementary data

1. Gels of enzymatic and chemical probing of SAF-93

Chemical and enzymatic probing and footprinting of prion aptamers. (A) Example of enzymatic probing of prion aptamer 93 using T1, V1 and S1 nucleases. Autoradiogram of 15% Urea-PAGE of 5'-labelled aptamer. Lane OH and G represent hydroxyl and nuclease T1 ladders, respectively. Gaps in the hydroxyl ladder indicate the positions of 2'-fluoro-pyrimidines that are resistant to alkaline hydrolysis. (B) Example of chemical probing of aptamer 93 with (DMS), dimethyl sulphate and (CMCT), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulphonate. Auto radiogram of 10% polyacrylamide gel of primer extension products using 5'-end-labelled oligonucleotide (AATTAACCCTCAC) complementary to the 3' end of aptamer 93. The aptamer was probed in 1 × HMKN buffer pH 7.2 for DMS and in 50 mM sodium borate pH 8.0 containing 10 mM MgCl2 and 50 mM KCl for CMCT. Reactions were carried out at 20°C for 5 and 20 minutes for DMS and CMCT, respectively. Unmodified (control lane) aptamer 93 was run in parallel to discriminate between stops specifically induced by chemical modifications and those due to the presence of stable secondary structures and false stops of AMV reverse transcriptase. Note that primer extension stops one residue prior to the modified bases, so the bands in the probing lanes are shifted down one residue relative to the corresponding sequencing bands. Lanes U, C, G and A are dideoxy sequencing ladder. (C) Footprinting of recombinant bovine alpha-PrP binding site onto aptamer 93 using nucleases T1, V1 and S1. Lane C, control 5'-end labelled aptamer; Lanes OH and G represent hydroxyl and RNase T1 ladders, respectively. The
black wedges at the top of the gel indicate the increasing concentrations (0, 120, 360 and 1080 nM) of alpha-PrP.

2. Footprinting gel of SAF-93(1-60).

Footprinting was carried out by incubating the RNA labelled on the 5′ end in the presence of increasing amounts of PrP in β form for 1 h at 25°C. Following incubation the RNA was digested with enzyme V1 RNase (5 x 10⁻³ units) which digests double-stranded regions, or S1 nuclease (0.05 units) which digests single stranded regions at 20°C for 5 min in HNMK buffer. The reaction was then stopped with sodium acetate and phenol/chloroform and ethanol precipitated. The RNAs were resuspended in formamide buffer and loaded on an 20% denaturing urea gel, along with a partial alkaline hydrolysate and RNase T1 digest (G residue ladder) to enable alignment to the known sequence. Bands attenuated at higher protein concentrations mark regions that are in contact with protein. SAF-93(1-60) is the essential binding region for PrP, and therefore the majority of RNA is protected from enzymatic digestion at the higher protein concentrations.
Sayer et al Figure 3
A figure from Sayer et al. shows the fraction bound as a function of [PrP] (nM) with two curves representing Beta and Alpha forms. The graph includes a Y-axis labeled 'Fraction Bound' ranging from 0.0 to 1.0 and an X-axis labeled '[PrP] (nM)' ranging from 1 to 1000.

Below the graph, there is a table labeled 'Beta Form, Alpha Form, H S A' with two rows and three columns, but the specific values are not legible.

An image showing a gel electrophoresis result is also included, with bands indicating the presence of different forms of PrP.
Structural determinants of conformationally selective, prion-binding aptamers
Natalie M. Sayer, Matthew Cubin, Alexandre Rhie, Marc Bullock, Abdessamad Tahiri-Alaoui and William James

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