The prion protein (PrP) is the major agent implicated in the diseases known as transmissible spongiform encephalopathies. The onset of transmissible spongiform encephalopathy is related to a change in conformation of the PrP (PrPSc), which loses most of its α-helical content, becoming a β-sheet-rich protein, known as PrPSc. Here we have used two Syrian hamster prion domains (PrP 109–141 and PrP 109–149) and the murine recombinant PrP (rPrP 23–231) to investigate the effects of anilino-naphthalene compounds on prion oligomerization and aggregation. Aggregation in the presence of bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate), ANS (1-anilinonaphthalene-8-sulfonate), and AmNS (1-amino-5-naphthalenesulfonate) was monitored. Bis-ANS was the most effective inhibitor of prion peptide aggregation. Bis-ANS binds strongly to rPrP 23–231 leading to a substantial increase in β-sheet content and to limited oligomerization. More strikingly, the binding of bis-ANS to full-length rPrP is diminished by the addition of nanomolar concentrations of oligonucleotides, demonstrating that they compete for the same binding site. Thus, bis-ANS displays properties similar to those of nucleic acids, causing oligomerization and conversion to β-sheet (Cordeiro, Y., Machado, F., Juliano, L., Juliano, M. A., Brentani, R. R., Foguel, D., and Silva, J. L. (2001) J. Biol. Chem. 276, 49400–49409). This dual effect of bis-ANS on prion protein makes this compound highly important to sequester crucial conformations of the protein, which may be useful to the understanding of the disease and to serve as a lead for the development of new therapeutic strategies.

The prion protein (PrP) is known to be responsible for the diseases called transmissible spongiform encephalopathies, which are invariably fatal, including scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and several human diseases such as Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnium (FFI) (1–3). The onset of a particular transmissible spongiform encephalopathy is caused by the conversion of the cellular PrP (PrPc), which is found predominantly on the outer surface of neurons attached to the membrane by a glycosyl phosphatidylinositol anchor, into an abnormal isoform, designated PrPSc (from Scrapie) (1). Prion diseases may present sporadic, inherited, or transmissible origins. However, in most cases the PrPSc has the same primary sequence as the PrPc, indicating that the conversion from one form to the other is not caused by a covalent modification (1–3). PrPSc has a high α-helical content, is markedly sensitive to proteolysis and is a soluble protein. On the other hand, the PrPSc has a high β-sheet content, displays insolubility in detergents and resistance to proteolysis in its aggregated or oligomeric forms (1–3).

To date, no other agent besides PrP has been identified to be implicated in such disease. However recent experimental evidence show that glycosaminoglycans (4–7) and nucleic acid molecules may be involved in PrPc-PrPSc conversion (8–11). A variety of compounds have been tested for their ability to prevent prion aggregation or to disrupt the aggregates (12), such as branched polyamines (13), Congo red and analogues (14–16), acridine and phenothiazines derivatives (17–19). Recent work from Kocisko et al. (20) describes a high-throughput screening assay for PrPSc inhibitors, from which they characterized one class of compound able to inhibit cell-free conversion reaction.

Aniline-naphthalene sulfonate derivatives, such as bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate) and ANS (1-anilinonaphthalene-8-sulfonate) were utilized in this work to test their effects on the oligomerization properties of prion proteins. Bis-ANS binds strongly to tubulin and inhibits its assembly into microtubules in vitro (21). Prevelige and co-workers (22) also showed that bis-ANS inhibits bacteriophage P22 capsid assembly. More recently, Bonafe et al. (23) showed that bis-ANS causes inactivation of vesicular stomatitis virus, probably by perturbing protein-protein interactions. Bis-ANS has been extensively used to probe conformational changes in proteins, because it binds to hydrophobic regions surrounded by positively charged residues (24–26). The structures of ANS, bis-ANS, and AmNS are shown in Fig. 1.

To investigate the effect of different hydrophobic compounds on prion peptide aggregation we have performed aggregation kinetic measurements in the presence of these naphthalene sul-
Mimicry of Nucleic Acid Binding to PrP by Amino-naphthalene Sulfonates

We used as models two peptides corresponding to Syrian hamster PrP (SHaPrP) residues 109–141 (PrP 109–141) and 109–149 (PrP 109–149) and the recombinant cellular murine PrP (rPrP 23–231). The peptides studied correspond to a loop and to the first α-helix of the N-terminal region of PrP, which is implicated in the conversion into β-sheets, and it is one of the most conserved regions in the PrP (28). The conversion into β-sheets is followed by aggregation into amyloid fibers (29). The peptide PrP (109–141) is of particular interest because structural data are available and its aggregation properties are well documented (29). The slightly longer peptide, PrP (109–149), was chosen because it contains a tryptophan residue at position 145 and it would be helpful for the fluorescence measurements. In mixed water/organic solvents and detergents, the peptide PrP (109–141) assumes a stable α-helical structure (29), and the random-coil structure is stable in the presence of high urea concentrations.

The aggregation of prion peptides was monitored by different spectroscopic measurements. The peptides were unfolded in buffer at pH 4.0–5.0 containing 6 M urea and were further diluted in the same buffer without the denaturant. Aggregation was monitored by the increase in light scattering at 320 nm as a function of time. On dilution in aqueous buffer, aggregation occurred within a few seconds (Fig. 2, A and B) and was markedly dependent on peptide concentration (Fig. 2C). We have performed circular dichroism measurements for both peptides in buffer containing SDS, confirming the data from the literature (29). Both peptides displayed α-helical secondary structure in sodium acetate buffer pH 4.0 in the presence of 100 mM SDS (data not shown).

PrP (109–141) peptide aggregated preferentially at pH 4.0, and PrP (109–149) similarly aggregated at pH 4.0 and pH 5.0. PrP (109–141) required higher concentrations than PrP (109–149) to achieve the same extent of aggregation (Fig. 2, A and B). PrP 109–141 at 10 μM aggregated less than PrP 109–149 at ten times lower concentration (1.0 μM) (Fig. 2, A and B). The emission fluorescence spectra of PrP (109–149) were collected and we observed that the tryptophan fluorescence was quenched upon aggregation (data not shown).

We tested whether the compounds bis-ANS and ANS could inhibit the prion peptide aggregation. Bis-ANS and ANS are aniline-naphthalene derived compounds (Fig. 1) that are well described for their ability to bind to hydrophobic regions on proteins, resulting in an increase in its fluorescence quantum yield upon binding to these pockets (24, 30, 31). The partially unfolded peptides were diluted in buffer containing varying concentrations of bis-ANS, and the aggregation was monitored by changes in light scattering. It was observed that bis-ANS inhibited prion peptide aggregation to a great extent at concentrations below 5 μM (Fig. 3) for both prion domains (data not shown).

**RESULTS**

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**EXPERIMENTAL PROCEDURES**

**Reagents**—All reagents used were of analytical grade. The compounds bis-ANS, ANS, and AmNS were purchased from Molecular Probes (Eugene, OR). The compound AmNS was purchased from Riedel-de Haen (Seelze, Germany). The 34-mer double-stranded DNA sequence (polGC) was synthesized and purified by Interactiva (Ulm, Germany).

**Peptide Synthesis and rPrP (23–231) Expression and Purification**—The prion peptides were kindly provided by Prof. M. A. Juliano and L. Juliano from Universidade Federal de Sao Paulo, Sao Paulo, Brazil, synthesized in solid phase. Purity of the peptides was assessed by mass spectrometry and reversed phase high-performance liquid chromatography. The peptides were maintained in a 6 M urea, 10 mM SDS stock solution that was diluted at least 100 times in the aggregation kinetic assays. The recombinant PrP (23–231) was expressed in Escherichia coli and purified by high-affinity column refolding as described previously (27). rPrP 23–231 histidine tag was cleaved by α-human thrombin (kindly provided by Prof. Robson Q. Monteiro, Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro), and rPrP was further purified as previously described (27). Peptides primary sequence: PrP (109–141), MKHMGAAAGAVVGGGLGYYMLGSMRPMHGFNDWEDER. AmNS, and ANS (bis-ANS and ANS) bind to the prion domains studied in this work. No aggregation inhibition mediated by ANS was observed at the drug concentrations tested, and although we could not detect binding of AmNS to both the peptides and the recombinant murine PrP, this compound inhibited prion peptide aggregation at higher concentrations. Interestingly, when we tested the effect of bis-ANS binding on the recombinant murine prion protein, this compound triggered protein oligomerization, but inhibited aggregation. These results suggest a dual role for bis-ANS in the aggregation process, highly dependent on protein domain and conformational state. We have recently demonstrated that both the prion peptides and the recombinant cellular PrP interact tightly with several plasminoids and double-stranded oligonucleotides (8), and we show here that bis-ANS competes with DNA for binding to the peptides and to the cellular prion protein. Similar to nucleic acid, bis-ANS leads to an increase in the content of β-sheet, usually observed when oligomerization occurs. The ability of a hydrophobic molecule to inhibit fibrillation of the whole protein makes it an excellent compound to explore the highly fluctuating structure of PrP.

**Spectroscopic Measurements**—Light scattering and fluorescence spectra were recorded on a PC1 spectrofluorometer (ISS, Champaign, IL), in "L" geometry (at 90° in relation to excitation light). Aggregation was followed as a function of light scattering (excitation 320 nm, emission 320 nm). For the aggregation kinetic assays, the peptides previously unfolded in a 5 or 6 M urea solution at pH 4.0 or 5.0 were diluted in buffer and light scattering was monitored as a function of time. Maximum light scattering obtained from each kinetic trace was measured and normalized considering control as 100% aggregation. The tryptophan fluorescence of rPrP (23–231) and of the peptide PrP (109–149) was measured by exciting at 280 nm and the emission verified from 300 to 420 nm. bis-ANS, ANS, and AmNS were excited at 360 nm, and fluorescence emission was collected from 400 to 600 nm for bis-ANS and from 400 to 650 nm for ANS and AmNS.

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**Fig. 1. Chemical structures of naphthalene derivatives.** A, bis-ANS; B, ANS; and C, AmNS.
shown for PrP 109–141). The aggregation inhibition was dependent on compound concentration (Fig. 3), but aggregation was not completely abolished even using higher concentrations of bis-ANS. We consider the possibility that a small fraction of the peptides in solution was aggregated prior to incubation with bis-ANS. The binding of the peptides to bis-ANS was also verified by the increase in fluorescence emission of the probe (Fig. 4, A and B; data not shown for PrP 109–141). The binding of the two peptides to the bis-ANS was very similar, indicating a 1 to 1 binding stoichiometry. If the bis-ANS is added to previously aggregated PrP (109–149), it is not able to bind to the same extent as observed upon adding the unfolded peptide to a bis-ANS solution. This result suggests that, after aggregation takes place, there are not enough exposed hydrophobic sites for probe binding, and this compound cannot reverse the aggregation reaction. We monitored the fluorescence emission spectra of a 2.0 μM solution of PrP (109–149) upon addition of bis-ANS (Fig. 5). This compound quenched the peptide tryptophan fluorescence, indicating proximity of bis-ANS binding site to the peptide tryptophan moiety.

We have monitored the effect of other naphtalene-derived compounds, ANS and AmNS, on prion peptide aggregation (Fig. 6, B and C). In contrast with the results obtained with bis-ANS, neither of these compounds could inhibit significantly PrP 109–149 aggregation at the concentrations tested. Only AmNS showed a minimal inhibition effect at concentrations greater than 50 μM (Fig. 6 C).

To further investigate the interaction of prion with bis-ANS, we studied the interaction of the recombinant murine prion protein (rPrP 23–231) with bis-ANS and verified the contribution of this hydrophobic compound to the aggregation profile of the whole prion protein as well. We have performed titration curves to calculate the average prion protein affinity to bis-ANS. As our data show (Fig. 7 A), rPrP appears to bind to bis-ANS over the same concentration range observed for the peptide (Fig. 3), suggesting that the binding regions in the

**Fig. 2. Aggregation of PrP (109–141) and PrP (109–149) at pH 4.0.** The unfolded peptides in 6 M urea, pH 4.0 were diluted in 50 mM sodium acetate (NaAc) at pH 4.0 in the presence (dashed line) or in the absence (solid line) of the denaturant (6 M urea). Light scattering values at 320 nm were measured as a function of time. A, PrP (109–141) at 10 μM; B, PrP (109–149) at 1.0 μM. The arrow indicates the moment of peptide dilution in the buffer. C, concentration dependence of PrP (109–149) aggregation. Relative light scattering is the higher light scattering value from an aggregation kinetic divided by initial light scattering value without peptide addition.
whole protein are located in the domain comprising amino acids 109–149. rPrP fluorescence intensity was also quenched in the presence of increased concentrations of bis-ANS, in agreement with the data obtained with prion peptide (Fig. 7B).

In contrast to the results obtained with the prion peptides, addition of bis-ANS to the full-length rPrP induced an increase in light scattering corresponding to the assembly into a small oligomer (Fig. 7A). There was only a limited increase in light scattering for rPrP in the presence of bis-ANS (≈3 times) in comparison with prion peptide aggregation (≈20 times) (Fig. 2A). In addition, the prion protein exhibited a high β-sheet content when bound to bis-ANS as verified by circular dichroism spectroscopy (Fig. 7C).

We have previously demonstrated that both the prion peptides and the recombinant cellular PrP interact strongly with several double-stranded oligonucleotides (8). Here we tested if nucleic acid and bis-ANS would compete for binding to prion peptide and to rPrP. Fig. 8 shows that addition of a double-stranded oligonucleotide to bis-ANS bound rPrP, displaces the hydrophobic compound, as seen from the decrease in bis-ANS fluorescence emission upon nucleic acid addition. The same result was obtained for PrP 109–149 (data not shown).

This dual effect of bis-ANS on prion protein makes this compound highly important to sequester crucial conformations of the protein, which may be useful to the understanding of the disease and to the development of new therapeutic strategies.

**DISCUSSION**

Here we describe that a hydrophobic compound (bis-ANS) can either inhibit aggregation of small prion domains or cause limited oligomerization of the full-length protein. These effects are very similar to those caused by nucleic acids, which we proposed could act as catalysts in the conversion of PrPC to PrPSc (8). This hypothesis has gained increasing attention with experimental evidence from other laboratories (9–11) and was recently strengthened by the demonstration that PrPres (PrPSc resistant to protease) is highly amplified after mixing infected and normal brain homogenates in the presence of nucleic acids (11, 32). The capacity of this very simple molecule, bis-ANS, to populate oligomeric conformations of the prion protein provides an useful new tool to study the prion conversion reaction.

Since the discovery of the transmissible spongiform encephalopathy agent there has been a great deal of effort expended to preventing or treating this disease, mainly by searching for compounds that could preclude the PrPC → PrPSc conversion. Unfortunately, no such molecule has proven to be efficient in combating this disease (18, 19). In the last years, several groups (17–19) have demonstrated that acridine and phenothiazine derivatives, especially quinacrine and chlorpromazine,
could clear PrPSc infection from ScN2a cultured cells chronically expressing PrP. But quinacrine did not prolong survival in a murine Creutzfeldt-Jakob disease model (33), and high doses of quinacrine generated liver damage without clearance of the disease in humans suffering from Creutzfeldt-Jakob disease (34).

Many current approaches in drug screening for prion diseases have focused on the identification of existing compounds that could be directly employed as a "second-use" approach, bypassing many clinical stages in drug approval. Screening for activity is mainly conducted by evaluating antagonization of prion propagation in cell and animal models of the disease, as it represents a more "realistic" approach than an in vitro cell-free system. However, questions concerning the suitability of therapy with compound screening to date is questionable, mainly due to limited ability to cross the blood-brain barrier (35). Recent work from Kocisko et al. (20) has shown that in a high-throughput screening assay for PrPSc inhibitors, of several compounds tested, some displayed high inhibition efficiency. A few of these new PrPSc inhibitors cross the blood-brain barrier.

Given their ability to inhibit prion peptide aggregation, the anilino-naphthalene compounds described here might serve as lead compounds with anti-prion activity. Despite the fact that bis-ANS promoted the β-sheet conversion of rPrP, its binding limited the aggregation to a large extent. Previous data from the literature have described that bis-ANS binds strongly to tubulin and potently inhibits its assembly into microtubules in vitro (21) and that it could also inhibit bacteriophage P22 capsid assembly (22). Early results from our research group also characterize naphthalene sulfonates, including bis-ANS, as inhibitors of β-amyloid peptide aggregation.2 ANS has been used as an indicator of protein aggregation as it is known to bind to hydrophobic surfaces in proteins and bis-ANS has been extensively used to probe conformational changes in proteins, as it binds to hydrophobic regions surrounded by positively charged residues (24–26).

Our previous work (8) has shown that the same prion domains studied here bind with high affinity to several double-stranded DNA sequences and that peptide aggregation was prevented upon binding to DNA. We showed here that bis-ANS competes with DNA for binding to the peptides and to the cellular recombinant prion protein. Moreover, potential compounds that would primarily act through disruption of hydrophobic interactions seem to be the most prominent, because it seems to be the force that commonly directs protein aggregates such as PrPSc, due to stabilization of intermolecular β-sheet structures (36, 37).

The mechanism by which the hydrophobic probe bis-ANS prevents prion peptide aggregation is not completely understood. However, our data indicate that binding of bis-ANS to the prion hydrophobic domains causes, among other effects, a steric impediment of contact between the peptides. Thus, when

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*2 A. D. Ferrão-Gonzales, personal communication.*
the peptide is bound to bis-ANS it cannot interact with another peptide molecule. The stoichiometry of the bis-ANS:PrP (109–149) binding was close to 1.0. Concerning the full-length PrP protein, the binding of bis-ANS seems to lead to larger conformational changes and oligomerization. Indeed, in future work we will attempt to characterize the structural properties of the peptide molecule. The stoichiometry of the bis-ANS:PrP (109–149) were recorded in a 2.00 mm path-length cell and were subtracted from the spectra. The formation of a folding intermediate with increased hydrophobic surface not observed in tetradecameric GroEL (38). Oligomeric species of α-synuclein (39) and of the amyloidogenic species of transthyretin (40) also bind anilino-naphtalene sulfonate compounds.

The other naphtalene-derived probes used (ANS and AmNS) were not as efficient in inhibiting aggregation as bis-ANS. Only AmNS showed minimum aggregation inhibition efficiency at concentrations above 50 μM. Considering that these molecules are similar in substituting groups and its positions, it seems that a crucial aspect of binding to prion protein and inhibition of aggregation because the contact of just one naphtalene ring to the peptide surface is not enough to prevent contact between other peptides. A more extended list of anilino-naphtalene sulfonate analogs with different derived pharmacophoric substituents is currently being tested for inhibition and disruption of prion aggregation.

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